# The role of *Fusobacterium necrophorum* in sheep and the environment in the severity and persistence of footrot

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# Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself under the supervision of my supervisors Professor Laura Green and Dr Kevin Purdy, and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author.

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## Summary

Ovine footrot is an infectious cause of lameness in sheep that has significant economic impact for the UK sheep farming industry. It is also a major concern for animal health and welfare. The causal agent is *Dichelobacter nodosus*, and *Fusobacterium necrophorum* is an opportunistic secondary pathogen that increases disease severity.

The primary reservoirs for *F. necrophorum* in sheep were believed to be sheep faeces and the environment, however, no studies had demonstrated the presence of *F. necrophorum* at either of these sites.

Two longitudinal studies (Study A and Study B) were conducted to determine reservoir sites of *F. necrophorum* in ovine footrot. Study A included 10 sheep sampled on four occasions at two week intervals. Study B included 40 sheep sampled weekly for 20 weeks. Samples collected from sheep and their environment were foot swabs, mouth swabs, faeces, soil and grass. Quantitative PCR was used to detect and quantify *F. necrophorum*. A multiple locus variable number tandem repeat analysis (MLVA) community typing scheme for *F. necrophorum* was developed and validated, and used to analyse samples from Study A and Study B.

Contrary to prior assumption, the environment was not a significant reservoir of *F. necrophorum. F. necrophorum* persisted in sheep, primarily on feet with footrot. MLVA indicated that the strains of *F. necrophorum* found on the feet of sheep were closely related, and they may therefore share characteristics that make them well adapted to feet and footrot. Mouths and faeces were an intermittent reservoir for the strains of *F. necrophorum* involved in footrot. Mouths and faeces may therefore facilitate persistence of *F. necrophorum* in the absence of footrot, or facilitate transmission of *F. necrophorum* between flocks. Mouths were a persistent reservoir for strains of *F. necrophorum* not involved in footrot.

# List of abbreviations

| | |
|---|---|
| AHDB | Agriculture and Horticulture Development Board |
| ATL | Tissue lysis buffer |
| AWERB | Animal Welfare Ethical Review Body |
| BBQ | Blackberry quencher |
| BLAST | Basic local alignment search tool |
| Bp | Base pairs |
| BSA | Bovine serum albumin |
| CS | Cross sectional |
| CI | Confidence interval |
| CODD | Contagious ovine digital dermatitis |
| Defra | Department for the Environment, Food and Rural Affairs |
| DNA | Deoxyribonucleic acid |
| *D. nodosus* | *Dichelobacter nodosus* |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| FAM | 6-carboxyl-fluorescein |
| FISH | Fluorescence in-situ hybridisation |
| *F. necrophorum* | *Fusobacterium necrophorum* |
| goeBURST | Global optimal eBURST algorithm |
| HGDI | Hunter Gaston Discriminatory Index |
| ID | Interdigital dermatitis |
| Kb | Kilobase |
| LB broth | Luria Bertani broth |
| MLST | Multilocus sequence typing |
| MLVA | Multiple locus variable number tandem repeat analysis |
| OR | Odds ratio |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| PFGE | Pulsed field gel electrophoresis |
| qPCR | Quantitative PCR |
| RAMS | Recto-anal mucosal swab |
| RNA | Ribonucleic acid |
| *S. aureus* | *Staphylococcus aureus* |
| SFR | Severe footrot |
| SLV | Single locus variant |
| Spp. | Species (multiple) |
| *S. uberis* | *Streptococcus uberis* |
| TDL | Theoretical detection limit |
| *T. pyogenes* | *Trueperella pyogenes* |
| UV | Ultraviolet |

# Chapter 1 General Introduction

## 1.1 Ovine footrot and its importance for sheep health and welfare

Footrot is an infectious dermatitis of the interdigital skin of sheep that causes lameness. This leads to poor welfare (Ley *et al.*, 1995; Goddard *et al.*, 2006), poor health and reduced productivity (Marshall *et al.*, 1991; Nieuwhof *et al.*, 2008; Wassink *et al.*, 2010), with resulting economic losses for sheep farmers. Footrot is reported in sheep farming countries worldwide, and in the UK it is the most common cause of lameness in sheep (Grogono-Thomas & Johnston, 1997; Kaler & Green, 2008; Winter *et al.*, 2015). It is one of the top three economically significant diseases for the UK sheep industry, with an estimated cost of between £24 and £80 million per annum (Nieuwhof & Bishop, 2005; Wassink *et al.*, 2010).

## 1.2 Aetiology and pathogenesis of footrot

There are two clinical presentations of footrot: interdigital dermatitis (ID), characterised by inflammation of the interdigital skin, and severe footrot (SFR) where hoof horn separates from the underlying sensitive tissue (Figure 1.1). The causal agent of footrot is the Gram-negative bacterium *Dichelobacter nodosus* (Beveridge, 1941; Kennan *et al.*, 2011; Witcomb *et al.*, 2014). Damage or softening of the interdigital skin, usually through wet conditions or rough pasture, facilitate infection (Beveridge, 1941; Graham & Egerton, 1968; Egerton *et al.*, 1969). Natural immunity to footrot is poor (Beveridge, 1941), and therefore sheep can get footrot repeatedly (Kaler *et al.*, 2010b). *D. nodosus* is transmitted between sheep via the environment (Beveridge, 1941; Whittington, 1995), and environmental conditions and management factors such as stocking density affect disease prevalence (Graham & Egerton, 1968; Wassink *et al.*, 2003; Wassink *et al.*, 2004; Kaler & Green, 2009). In the UK footrot occurs year-round due to the mild, wet climate (Green & George, 2008; Ridler *et al.*, 2009; Smith *et al.*, 2014), however, in areas such as Western Australia with Mediterranean type climates, footrot occurrence is seasonal (Graham & Egerton, 1968; Depiazzi *et al.*, 1998).


**Figure 1.1 Clinical presentations of footrot.**
A: Healthy foot; B: Interdigital dermatitis (ID) with inflammation visible in the interdigital skin; C: Severe footrot (SFR) with separation of the sole horn and necrotic tissue.

## 1.3 Current footrot situation in England

The prevalence of lameness in sheep in England in 2013 was estimated to be 5% (Winter *et al.*, 2015), a decrease from the 10% reported in 2004 (Kaler & Green, 2008). Over 90% of sheep farmers in England report that there is footrot in their flock, and farm level prevalence varies: the median prevalence of ID and SFR reported in 2013 were 5% and 3% respectively, but the distributions are highly skewed with the maximum prevalence reported being 90% for ID and 40% for SFR (Winter *et al.*, 2015).

The current recommended treatment for footrot is administration of a long acting systemic antibiotic together with topical antibiotic treatment to all four feet, within three days of onset of lameness (Kaler *et al.*, 2010a; Wassink *et al.*, 2010). Where this practice is used consistently, lameness prevalence reduces to < 2% (Wassink *et al.*, 2010). Whole flock management strategies including biosecurity, vaccination, culling and genetic selection are also used to control footrot. Flock level elimination of footrot has been achieved in several countries (Egerton *et al.*, 2002; Gurung *et al.*, 2006; Mills *et al.*, 2012; Forbes *et al.*, 2014; Greber *et al.*, 2016), however, the frequent movement of sheep between farms in England makes reintroduction of disease a significant risk and therefore elimination is not feasible except in closed flocks (Green & George, 2008; Clifton & Green, 2016).

## 1.4 Characterisation of *Fusobacterium necrophorum*

*Fusobacterium necrophorum* is a Gram-negative, pleomorphic, generally rod-shaped anaerobe that uses lactate as its main energy substrate (Lechtenberg *et al.*, 1988). It is one of fourteen species of the genus *Fusobacterium* (Shah *et al.*, 2009), and is a pathogen of both humans and animals (Nagaraja *et al.*, 2005). There are two subspecies of *F. necrophorum: F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* (Shinjo *et al.*, 1991). *F. necrophorum* subsp. *necrophorum* is more pathogenic (Nagaraja *et al.*, 2005) and is more commonly found in animal disease whereas *F. necrophorum* subsp. *funduliforme* is more frequently reported in human disease (Hall *et al.*, 1997).

### 1.4.1 *Fusobacterium necrophorum* and disease

*F. necrophorum* is an opportunistic pathogen with reservoirs (sites in living organisms or the environment where bacteria live and usually multiply) in healthy individuals. It causes diseases characterised by necrotic lesions and abscesses, termed necrobacilloses (Langworth, 1977; Tan *et al.*, 1996). Hepatic abscesses in cattle and pharyngotonsillitis in humans are the most well studied examples and these are detailed below. *F. necrophorum* is also associated with other diseases including periodontal disease in wallabies, calf diphtheria, digital necrobacillosis in ungulates and endometritis in cattle (Ruder *et al.*, 1981; Monrad *et al.*, 1983; Panciera *et al.*, 1989; Edwards *et al.*, 2001; Antiabong *et al.*, 2013b; Aghamiri *et al.*, 2014).

#### 1.4.1.1 Hepatic abscesses in cattle

Hepatic abscesses are an example of the opportunistic behaviour of *F. necrophorum*. *F. necrophorum* is present in the rumen of cattle (Tan *et al.*, 1994; Narayanan *et al.*, 1997), and following damage to the rumen wall the bacterium invades the portal circulation and is transported to the liver where it can result in abscess formation. Evidence for this pathogenesis was provided through identification of identical ribotypes of *F. necrophorum* in the rumen and liver of the same individual (Narayanan *et al.*, 1997).

#### 1.4.1.2 Pharyngotonsillitis in humans

Recently *F. necrophorum* has been highlighted as a significant cause of pharyngotonsillitis in humans (Eaton & Swindells, 2014; Jensen *et al.*, 2015; Holm *et al.*, 2016). It was originally believed that *F. necrophorum* was present as a commensal in the throat of healthy individuals of all ages, however, recent evidence indicates that it is primarily present in adolescents and young adults, with *F. necrophorum* pharyngotonsillitis also being more prevalent in this age group (Aliyu *et al.*, 2004; Jensen *et al.*, 2007; Ludlam *et al.*, 2009; Jensen *et al.*, 2015; Van *et al.*, 2017). Social behaviour is one suggested explanation for this age distribution: Ludlam *et al.* (2009) demonstrated an association between *F. necrophorum* colonisation and a history of lip-to-lip kissing contacts in the previous 4 weeks. This finding suggested that *F. necrophorum* is transmitted between individuals. An alternative suggestion is that the observed age distribution is related to changes in immune status and the onset of tonsillar atrophy around puberty (Holm *et al.*, 2016).

In some cases, *F. necrophorum* pharyngotonsillitis may progress to Lemierre's syndrome, a severe disease that can be fatal if left untreated. The pathogenesis involves invasion of the jugular vein and haematological spread of *F. necrophorum* followed by metastatic abscess formation (Lemierre, 1936; Riordan, 2007).

### 1.4.2 Virulence factors of *Fusobacterium necrophorum*

*F. necrophorum* has a variety of virulence factors of which leukotoxin is the most well studied; the others are summarised in Table 1.1. Leukotoxin is considered to be the major virulence factor for disease in animals (Nagaraja *et al.*, 2005). It is a secreted protein that is cytotoxic to bovine leukocytes, causing cellular activation and apoptosis at low concentrations and necrotic cell death at high concentrations, a feature that may enable it to modulate the host immune response (Narayanan *et al.*, 2002). The role of *F. necrophorum* virulence factors in ovine footrot has not been investigated.

**Table 1.1 Virulence factors of *Fusobacterium necrophorum***

| Virulence factor | Role in pathogenesis | Difference between subspecies[a] | Reference |
|---|---|---|---|
| Haemagglutinin | Agglutination of erythrocytes | Fnf shows only weak agglutination | (Langworth, 1977; Shinjo & Kiyoyama, 1986; Horose *et al.*, 1992; Hall *et al.*, 1997) |
| Haemolysin | Cytotoxic to mammalian cells<br>Iron acquisition | Fnn is more haemolytic than Fnf | (Amoako *et al.*, 1994; Amoako *et al.*, 1996; Amoako *et al.*, 1998) |
| Outer membrane proteins | Adherence to eukaryotic cells | Proteins differ between subspecies | (Kumar *et al.*, 2013) |
| Endotoxin | Protect bacteria from immune response<br>Highly immunogenic | Effects only described in Fnn | (Horose *et al.*, 1992; Garcia *et al.*, 2000) |
| Collagenolytic substance (CCWC) | May contribute to tissue necrosis | No data available | (Okamoto *et al.*, 2005) |

[a] Fnf = *F. necrophorum* subsp. *funduliforme*; Fnn = *F. necrophorum* subsp. *necrophorum*

## 1.5 The role of *Fusobacterium necrophorum* in ovine footrot

*Fusobacterium necrophorum* has been known to play a role in ovine footrot for over a century, however, the nature of this relationship has been re-evaluated several times. In the early 20$^{th}$ Century, *F. necrophorum* was believed to be the causal agent of ovine footrot (Mohler & Washburn, 1904). In 1941, Beveridge identified *Dichelobacter nodosus* as the causal agent. When sheep feet were inoculated with *D. nodosus,* footrot developed, however, when sheep feet were inoculated with pure cultures of *F. necrophorum*, the resulting lesions did not resemble footrot. Beveridge concluded that *F. necrophorum* was likely to be a secondary invader in footrot, and that it could increase lesion severity.

In 1969, Roberts and Egerton studied the aetiology and pathogenesis of footrot and suggested that *F. necrophorum* was essential for the development of footrot. They injected *D. nodosus* alone or with *F. necrophorum* into the interdigital skin of sheep that

were subsequently kept on wet mats, and observed that 1/11 sheep given *D. nodosus* alone developed footrot lesions compared to 8/11 given both bacteria. They subsequently applied *D. nodosus* cultures to the feet of sheep that had already been kept in wet conditions where faecal contamination was minimised, and sheep exposed to heavy faecal contamination. They observed that there was no evidence of footrot or *D. nodosus* colonisation in the sheep kept in clean conditions, but that footrot with invasion of *D. nodosus* and *F. necrophorum* occurred in 2/3 sheep kept in contaminated conditions. They concluded that a factor provided by faecal contamination was required for *D. nodosus* invasion, and that this factor was *F. necrophorum*. It is however possible that standing in pens heavily contaminated by faeces caused more damage to the interdigital skin than wet conditions alone, making feet more susceptible to invasion; the authors observed that only mild inflammation was present in skin sections taken from sheep kept in clean pens. It is also highly likely that if *F. necrophorum* were present in the faecal material, the constant exposure of the interdigital skin to this material would result in colonisation by *F. necrophorum*, however this does not reflect natural infection in sheep kept at pasture.

In a second study, Egerton *et al.* (1969) performed histological studies of natural footrot lesions, and observed that *D. nodosus* predominated in early lesions but that *F. necrophorum* predominated in later lesions. They also observed that severe inflammation was usually associated with *F. necrophorum*. In contrast, they observed that in lesions artificially induced with material taken from feet with footrot, *F. necrophorum* invaded the epidermis several days before *D. nodosus*. They concluded that *F. necrophorum* initiated invasion of the interdigital skin, facilitating colonisation by *D. nodosus,* and increasing lesion severity. The evidence from the natural lesions would suggest, however, that during natural disease progression *D. nodosus* invasion occurred prior to colonisation with *F. necrophorum,* and that *F. necrophorum* then increased lesion severity.

Recent evidence supports the theory that *D. nodosus* colonisation occurs prior to colonisation with *F. necrophorum*. In a study examining load of *D. nodosus* and *F. necrophorum* over time during natural infection, Witcomb *et al.* (2014) found an increase in load of *D. nodosus* before and during an episode of ID and prior to occurrence of SFR. In contrast, the load of *F. necrophorum* only increased once SFR had occurred. The authors concluded that if it is load that drives the pathogenesis of the disease, *D. nodosus* initiates disease and *F. necrophorum* is an opportunist once disease has occurred. Subsequent cross-sectional studies have also demonstrated highest prevalence and load of *D. nodosus* on feet with ID, and highest prevalence and load of *F. necrophorum* on feet with SFR (Witcomb *et al.*, 2015; Maboni *et al.*, 2016). The role of *D. nodosus* in driving pathogenesis of footrot has been recently demonstrated in a mathematical model (Atia *et al.*, 2017).

The suggested role for *F. necrophorum* as a secondary opportunist in ovine footrot is consistent with its opportunistic nature in other diseases (Section 1.4 above). In many conditions, *F. necrophorum* is thought to act synergistically with other bacterial pathogens to enhance the disease severity, for example in calf diphtheria, ovine foot abscesses and bovine hepatic abscesses (Roberts, 1967; Takeuchi *et al.*, 1983; Panciera *et al.*, 1989). During their histological studies of natural footrot lesions, Egerton *et al.* (1969) observed that *D. nodosus* predominated at the point of separation of the hoof horn but was associated with very little inflammation whilst *F. necrophorum* was associated with severe inflammation and tissue sloughing. They concluded that the characteristic separation of hoof horn in footrot is caused by *D. nodosus*, whilst the concurrent inflammation and necrosis are attributable to *F. necrophorum*.

## 1.6 The role of other bacterial species in ovine footrot

*D. nodosus* and *F. necrophorum* are not the only bacterial species to have been associated with ovine footrot. Roberts and Egerton (1969) demonstrated that inoculation of feet with a mixture of *D. nodosus*, *F. necrophorum* and *Trueperella pyogenes* increased the number of feet that became colonised with *D. nodosus* compared to when *T. pyogenes* was not included. Synergism between *F. necrophorum* and *T. pyogenes* has been reported

(Roberts, 1967; Ruder *et al.*, 1981; Takeuchi *et al.*, 1983), and *F. necrophorum* is frequently present in mixed infections with *T. pyogenes* (Tan *et al.*, 1996). It is thought that synergism with facultative bacteria may contribute to the establishment of an anaerobic environment for *F. necrophorum* (Roberts & Egerton, 1969), and that the leukotoxin from *F. necrophorum* may offer protection to other species (Takeuchi *et al.*, 1983). This organism has however not been included in recent studies of bacterial prevalence and load in ovine footrot (Witcomb *et al.*, 2014; Frosth *et al.*, 2015; Maboni *et al.*, 2016).

There has been recent interest in the role of *Treponema* spp. in ovine foot disease, and three phylogroups ("*Treponema medium*/*Treponema vincentii*-like", "*Treponema phagedenis*-like" and *Treponema pedis*) have been associated with contagious ovine digital dermatitis (CODD) in sheep (Sullivan *et al.*, 2015). This disease is characterised by ulceration of the skin at the coronary band, which is followed by separation of the hoof horn from the underlying tissue (Naylor *et al.*, 1998). During early studies of footrot, Beveridge (1941) and Egerton *et al.* (1969) observed spirochaetal organisms in footrot lesions. More recently, Frosth *et al.* (2015) detected *Treponema* spp. on the feet of sheep in Swedish flocks with and without footrot, but found no association between detection of these organisms and disease status of the flock. However, there are currently no longitudinal studies investigating variation in load of *Treponema* spp. during footrot. This type of study might further our understanding of the role of *Treponema* spp. as has been the case for *D. nodosus* and *F. necrophorum* (Witcomb *et al.*, 2014).

Beveridge (1941) observed that footrot lesions contain a great variety of bacteria, and that two organisms, a motile fusiform and *Spirochaeta penortha*, were frequently seen in large numbers in active lesions. In 2011, Calvo-Bado *et al*. used sequencing to study the microbial community of the ovine interdigital skin, and demonstrated that differences in this community could be seen between healthy feet and those with footrot. In particular, *Peptostreptococcus* were associated with healthy feet, *Corynebacterium* with ID, and *Staphylococcus* with SFR. These findings suggest that changes in the microbial community

of the foot occur in association with footrot, however, because this study was cross sectional the timescale of these changes in relation to disease progression is not clear.

Human periodontal disease is a well-studied example of a polymicrobial disease, with differences seen in the periodontal microbial community between healthy and diseased states (Moore *et al.*, 1982; Abusleme *et al.*, 2013). Early theory suggested that certain pathogens were important in the aetiology of disease and were therefore only present, or present in increased numbers, in disease (Loesche, 1976; Hajishengallis *et al.*, 2012; Nobbs *et al.*, 2013). In 1986, Theilade proposed that it is multiple species within a community, rather than individual species, that contribute to disease pathogenesis. A more recent theory suggests that dysbiosis of the microbial community (changes in relative abundance of members of the community) is initiated by 'keystone' pathogens present at low abundance (Hajishengallis *et al.*, 2012). These authors highlight *Porphyromonas gingivalis* as an example of a keystone pathogen because despite being present at low abundance, it can subvert the host immune response resulting in both alterations to the microbial community and periodontitis (Hajishengallis *et al.*, 2011). Research into the pathogenesis of ovine footrot has focused on the role of *D. nodosus* and *F. necrophorum*, and to-date little consideration has been given to the role of the microbial community and less abundant species.

## 1.7 Potential reservoirs of *Fusobacterium necrophorum* in sheep and their environment

It is believed that sheep faeces are the main source of *F. necrophorum* in footrot (Tan *et al.*, 1996). Roberts and Egerton (1969) observed colonisation of the interdigital skin by *F. necrophorum* when sheep were kept in pens heavily contaminated by faeces, as discussed above (Section 1.5), however they did not actually test faecal samples from sheep for the presence of *F. necrophorum*. In a recent study using molecular methods, Witcomb (2012) failed to detect *F. necrophorum* in sheep faeces collected directly from sheep but did detect *F. necrophorum* in 4/35 faecal samples collected from the floor of sheep pens and 2/7 faecal samples collected from the interdigital space of sheep. This may represent

contamination of faeces in the interdigital space or the environment by *F. necrophorum* present on the feet of sheep. Alternatively, faecal shedding of *F. necrophorum* in sheep faeces maybe intermittent, and therefore may not have been detected by Witcomb (2012) in samples taken from sheep because of the small number of animals studied at one time point (n=20).

*F. necrophorum* has been shown to survive in soil microcosms in the laboratory (Garcia *et al.*, 1971). Garcia *et al.* (1971) used a fluorescently-labelled antibody to visualise *F. necrophorum* cells in soil kept under a variety of conditions. They observed that antigenically reactive cells were still visible after 10 months in soil kept under anaerobic conditions at 4°C, and after 8 months at 37°C. Using acridine orange staining, they demonstrated survival of viable *F. necrophorum* cells for up to 8 weeks. This paper led to an assumption that *F. necrophorum* is ubiquitous in the environment of sheep and other ungulates, and this has been widely reported in reviews, the introductions of primary research papers and veterinary textbooks e.g. (Langworth, 1977; Winter, 2004b; Yeruham & Elad, 2004; Green & George, 2008; Dubreuil & Anderson, 2009; Handeland *et al.*, 2010; Underwood *et al.*, 2015). All the microcosms used by Garcia *et al.* (1971) were maintained at 80% water holding capacity, and therefore cannot provide information regarding the effect of different soil moisture levels on *F. necrophorum* survival. The microcosms were also held under constant conditions, whereas the conditions on sheep pasture will be much more variable. These experiments may therefore reflect the maximum survival of *F. necrophorum* under ideal conditions, but these conditions are unlikely to be maintained on sheep pasture.

The only study so far to test soil from sheep pasture for presence of *F. necrophorum* was a pilot study by Witcomb (2012). The 20 soil samples collected from one sheep pasture in this study were negative for *F. necrophorum* DNA. In other ungulates there are reports of outbreaks of necrobacillosis associated with animals congregating at feeding or watering areas, in periods of increased rainfall (Edwards *et al.*, 2001; Handeland *et al.*, 2010) and

heavily faecally contaminated pens (Monrad *et al.*, 1983). It is therefore possible that high stocking density of animals and suitable climatic conditions lead to transiently increased presence and survival of *F. necrophorum* in localised areas of pasture, however, these areas will always be contaminated with faeces. Other than Witcomb (2012) pilot data there are no soil samples analysed for the presence of *F. necrophorum* and grass has never been tested for presence of *F. necrophorum*.

*F. necrophorum* has been detected in the mouths of sheep (McCourtie *et al.*, 1990; Bennett *et al.*, 2009; Witcomb, 2012). Witcomb (2012) reported that 74% (26/35) of mouth swabs were positive for *F. necrophorum*, and also provided evidence from fluorescence in-situ hybridisation (FISH) studies that intact, physiologically active *F. necrophorum* were present in the oral cavity of sheep.

*F. necrophorum* is detected on healthy feet of sheep, and on feet with ID, but with lower frequency of detection and at lower loads than feet with SFR (Witcomb *et al.*, 2014; Frosth *et al.*, 2015; Maboni *et al.*, 2016). There are no data on the persistence (duration of carriage) of *F. necrophorum* on healthy feet.

## 1.8 The persistence of pathogens in endemic infectious diseases

### 1.8.1 Definitions of pathogen persistence

Detection of bacteria at a site might indicate transient contamination, transient colonisation or persistence. Persistence of pathogens in hosts and, for some pathogens, the environment is important for persistence of disease in populations (Krämer *et al.*, 2010).

In bacterial ecology, persistence within individual populations of bacteria can be achieved through a small pool of persister cells which exist in a slow-growing, stress tolerant state (Patra & Klumpp, 2013). The most frequently used example is the response of bacterial populations to antibiotics (Patra & Klumpp, 2013; Brauner *et al.*, 2016). In

metapopulations of bacteria, bacterial dispersal can exploit transient differences between sites (Holt, 1993): if conditions at one site become unfavourable for survival, the metapopulation is maintained by survival of a population at a different site where conditions are more favourable. The metapopulation idea is useful when considering persistence of pathogens in hosts or their environment.

Definitions of pathogen persistence used in epidemiological studies can be variable, for example Mehraj *et al.* (2016) highlight that persistent human carriers of *Staphylococcus aureus* have been defined based on the proportion of positive nasal swabs out of the number of swabs taken, however, the intervals between sample collection vary, different thresholds are applied, and some studies include strain types in their definitions whereas others do not. It is therefore unclear whether these data represent evidence of persistence, and it can be difficult to make comparisons between persistence in different studies. There are several studies of the persistence of *Escherichia coli* O157 on cattle farms, however, none of these define persistence but describe the number of consecutive positive samples from different locations (Shere *et al.*, 1998; Renter *et al.*, 2003; LeJeune *et al.*, 2004; Liebana *et al.*, 2005). Persistence requires repeated detection of an organism at a specific site over time, and a definition of persistence should therefore include how many consecutive detections are deemed to represent persistent rather than transient detection. It should also be considered how many consecutive negative samples are required to determine the end of a period of persistence, for example one negative sample amongst a series of positive samples could be deemed a false negative, and this needs to be included in the definition.

### 1.8.2 Examples of persistence strategies in bacterial pathogens

Reservoirs of bacterial pathogens are sites in an organism or the environment where the pathogen lives, and often multiplies (Krämer *et al.*, 2010). Endemic bacterial pathogens of livestock use a variety of reservoir sites in order to persist within a host or its environment. *Streptococcus uberis*, a common mastitis pathogen in dairy cattle, was detected in over 60% of environmental samples during a longitudinal study on one dairy

farm, and was detected repeatedly at the same environmental site over time (Zadoks *et al.*, 2005). In the same study *S. uberis* was also detected in cattle faeces, but cows very rarely had more than one faecal sample positive suggesting that *S. uberis* was not persistently shed in faeces. In contrast, *E. coli* has been detected in repeated faecal samples from both sheep and cattle (Shere *et al.*, 1998; Sanchez *et al.*, 2009), suggesting that the bacteria may persist within the GI tract. In a study of reservoir sites for *S. aureus* in sheep, *S. aureus* was detected in 57% (169/298) of nasal cavity swabs from ewes and 58% (171/294) of nasal cavity swabs from lambs (Mork *et al.*, 2012). This study did not describe persistent nasal carriage in individual sheep, however persistent nasal carriage of *S. aureus* has been reported in other species including pigs (Gibbons *et al.*, 2013) and humans (Eriksen *et al.*, 1994; Verhoeven *et al.*, 2012). Several of these studies provided evidence for transmission from reservoir sites, for example Shere *et al.* (1998) used longitudinal data to illustrate detection of the same strain of *E. coli* in drinking water following detection in faeces. Similarly Mork *et al.* (2012) identified the same strain types of *S. aureus* in ewes and their lambs. However, none of these studies looked at associations between persistence at reservoir sites and disease prevalence in the host population.

### 1.8.3 Persistence of *Fusobacterium necrophorum* in ovine footrot

*F. necrophorum* populations could persist on the feet of sheep, in the mouth, in faeces or on pasture. Persistence of *F. necrophorum* in a flock of sheep could occur as *F. necrophorum* move between sites within and between sheep. On a larger scale, the movement of sheep between flocks could facilitate persistence. There are no further data available on the persistence of *F. necrophorum* in sheep other than the limited data discussed in Section 1.7.

## 1.9 Associations between reservoir sites of *Fusobacterium necrophorum* and footrot

It is known that feet with footrot have an increased load of *F. necrophorum* (Witcomb *et al.*, 2014; Witcomb *et al.*, 2015). One can hypothesise that increased prevalence of footrot

will result in increased shedding of *F. necrophorum* into the environment and spread to other feet and sheep, however, this has not been demonstrated. This effect could also be increased when footrot lesions are of greater severity or duration, but again no studies have investigated this. Increases in *F. necrophorum* load on feet occur subsequent to increased loads of *D. nodosus* (Witcomb *et al.*, 2014), and *D. nodosus* transmission is known to occur via pasture (Whittington, 1995). It is possible that the two bacteria share transmission pathways, but associations between *D. nodosus* load and *F. necrophorum* load at any of the sites other than feet have not been investigated.

## 1.10 Methods for studying the epidemiology and ecology of *Fusobacterium necrophorum*

### 1.10.1 The use of longitudinal studies to understand persistence and transmission

Longitudinal studies involve measuring the outcome of interest repeatedly over time. They are valuable in epidemiology as they can provide evidence for causal associations, and they also facilitate investigation of persistence through detection of the same organism at the same site on repeated occasions. A longitudinal study is necessary to determine reservoirs of *F. necrophorum* in sheep and their environment, and their relevance to development, severity and chronicity of footrot. In this study, persistence will be defined as detection of *F. necrophorum* on two or more consecutive sampling occasions, and the duration of persistence will be defined as the time from the first positive sample until a negative sample occurs.

### 1.10.2 The use of bacterial load to study microbial ecology

The study by Witcomb *et al.* (2014) was the first to examine the load of *F. necrophorum* on feet. As highlighted by those authors, load, measured by quantitative PCR (qPCR), can provide a more detailed indication of changes in bacterial populations than simple presence/absence data provided by standard PCR, and when measured over time, load is an extremely useful epidemiological tool. Changes in bacterial pathogen load measured by qPCR have been shown to correlate to disease severity: in humans, patients with higher loads of *Mycoplasma pneumoniae* in oral secretions were more likely to be hospitalised

for pneumonia than those with lower loads (Nilsson *et al.*, 2010). Similarly patients admitted with severe meningitis had higher loads of meningococcal bacteria in their blood than those with mild disease (Hackett *et al.*, 2002). Bacterial load may also be associated with persistence: Verhoeven *et al.* (2012) used load of *S. aureus* on nasal swabs from humans, as measured by qPCR, to predict persistent nasal carriage.

### 1.10.3 The use of strain typing to understand persistence and transmission

The use of species level data is not sufficiently robust to understand persistence and transmission of bacterial pathogens, and therefore strain typing is essential in these types of epidemiological studies. Strain typing has been widely used to understand the epidemiology and ecology of bacterial pathogens in livestock, using a range of methods including ribotyping, pulsed field gel electrophoresis (PFGE), multiple locus variable number tandem repeat analysis (MLVA) and multilocus sequence typing (MLST) (Shere *et al.*, 1998; Zadoks *et al.*, 2005; Vranckx *et al.*, 2011; Davies *et al.*, 2016). Ribotyping and determination of sequence variation of the leukotoxin gene have been used to differentiate strains of *F. necrophorum* (Narayanan *et al.*, 1997; Zhou *et al.*, 2009), but understanding of the variability of *F. necrophorum* strains beyond the subspecies level is minimal.

### 1.10.4 Sampling methods for sheep and their environment

Swabs are used to sample the microbial community of the skin in both humans and animals (Gao *et al.*, 2010; Verdier-Metz *et al.*, 2012), and have been previously used in studies of ovine footrot (Moore *et al.*, 2005; Hill *et al.*, 2010; Witcomb *et al.*, 2014; Frosth *et al.*, 2015). Swabs are used for both culture of bacteria and molecular analyses, such as qPCR. Swabs are preferable to invasive methods such as biopsies because they minimise harm to the subject and do not change the course of the disease process, which is important in longitudinal studies (Witcomb *et al.*, 2014). Swabs are also commonly used to sample the microbial community of the oral cavity. They have been used to detect and quantify *F. necrophorum* from the human pharynx (Aliyu *et al.*, 2004; Jensen *et al.*, 2007), and from the gingival margin of wallabies (Antiabong *et al.*, 2013b).

Faecal sampling can be achieved by collection of a rectal faecal sample (Liebana *et al.*, 2005; Sanchez *et al.*, 2009), or a recto-anal mucosal swab (RAMS) (Rice *et al.*, 2003; Spencer *et al.*, 2015). RAMS was reported to be more sensitive for detection of *E. coli* O157 in cattle when using culture based methods, and distinguished colonisation from shedding (Rice *et al.*, 2003). If the purpose of a study is to understand transmission from reservoir sites, information regarding shedding may be more useful than colonisation.

Many approaches have been used to sample the environment of farm animals to detect bacterial pathogens. For pathogens where a particular mode of transmission is known to be important, sampling can be targeted to a certain degree to known high-risk areas. However, it is also often necessary to gain a representative overview of organisms present across the whole study area using systematic sampling grids (Brown *et al.*, 2004), stratified sampling (Nightingale *et al.*, 2004; Raizman *et al.*, 2004; Kersh *et al.*, 2013) boot swabs (Berghaus *et al.*, 2013; Eisenberg *et al.*, 2013) or a combination of methods (Marin & Lainez, 2009).

## 1.11 Summary and conclusions from current knowledge

*F. necrophorum* is a secondary pathogen in ovine footrot that may increase severity of footrot. It has been widely stated that *F. necrophorum* is ubiquitous on pasture, but there is very little evidence to substantiate this. *F. necrophorum* has been detected in mouths, healthy feet, and feet with footrot, and is suggested to be shed in sheep faeces, however, it is unknown which of these sites are reservoirs for *F. necrophorum*. It is also unknown whether changes in load of *F. necrophorum* at these sites are associated with changes in prevalence, severity or chronicity of footrot, or changes in load of *D. nodosus*.

## 1.12 Hypotheses

The aim of this thesis was to test the following hypotheses:

1. *F. necrophorum* is present in the environment of sheep (feet, mouth, faeces, soil, grass) and persists at these sites.

2. *F. necrophorum* load at these sites increases following increased incidence and prevalence of footrot in feet and sheep.
3. *F. necrophorum* load at these sites is higher with increasing severity and chronicity of footrot in feet and sheep.
4. *F. necrophorum* load at these sites increases subsequent to increased load of *D. nodosus*.

In addition, the following objectives were developed:

1. To develop and validate a multiple locus variable number tandem repeat analysis (MLVA) typing scheme for *F. necrophorum*.
2. To use this scheme to analyse communities of *F. necrophorum* in samples from longitudinal studies of sheep and their environment.

## 1.13 Thesis structure

In this thesis Chapter 2 outlines laboratory methods used to detect and quantify *F. necrophorum*. Chapter 3 describes two longitudinal studies (Studies A and B), designed to investigate persistence of *F. necrophorum* in sheep and their environment in two different climates. Chapter 4 (accepted for publication in Veterinary Microbiology) describes the development and validation of a multiple locus variable number tandem repeat analysis (MLVA) typing scheme for *F. necrophorum*, and includes MLVA analysis of samples collected during Study A. Chapter 5 presents the MLVA analysis of samples from Study B. Chapter 6 presents the overall discussion of the findings from this thesis.

# Chapter 2 Laboratory methods used for the detection and quantification of *Fusobacterium necrophorum*

This chapter details the laboratory methods used to detect and quantify *F. necrophorum* in samples collected from sheep (foot swabs, mouth swabs and faecal samples), and in environmental samples collected from sheep pasture (soil and grass).

## 2.1 DNA extraction from sheep and environmental samples

### 2.1.1 DNA extraction from environmental and faecal samples

DNA extraction from soil, faeces and grass was performed using the method described by Purdy (2005). This method involves chemical (phenol), detergent (sodium dodecyl sulphate) and mechanical (bead beating) cell lysis, followed by extraction and then purification of DNA on hydroxyapatite and then Sephadex spin columns. DNA is then ethanol precipitated. The centrifugation times for the ethanol precipitation stage were increased from 5 to 30 minutes.

The weight of each sample was recorded prior to extraction. There was approximately 0.5g of soil and grass and 0.1g of faeces per sample. Samples were processed in batches of 16 including an extraction blank (500µl sterile phosphate buffered saline (PBS)) processed in each batch as a negative control. The DNA extracted was re-suspended in 50µl sterile 10mM Tris pH 7.5 and stored at -20°C.

### 2.1.2 DNA extraction from swabs

The hydroxyapatite spin column method (Purdy, 2005) was used to extract DNA from swabs. Swabs were thawed before processing, and then transferred to a sterile 2ml screw-cap microcentrifuge tube using sterile tweezers and any PBS remaining in the cryotube was added to the tube. The extraction was then performed as described for environmental samples. Samples were processed in batches of 16 including an extraction

blank (500µl sterile PBS) processed in each batch. The DNA extracted was re-suspended in 50µl sterile 10mM Tris pH 7.5 and split into two 25µl aliquots and then stored at -20°C.

The only exception to this procedure was foot swabs from the five farms in the cross-sectional study (Section 4.3.6) because the samples were shared between two projects. In this case, swabs were centrifuged at 1600 ×*g* for 8 minutes to collect as much of the liquid soaked into the swab as possible. This liquid, with PBS remaining in the cryotube, was centrifuged at 13,000 ×*g* for 5 minutes and the supernatant discarded. The pellet was then re-suspended in 500µl sterile PBS, and 250µl were used in the subsequent DNA extraction.

### 2.1.3 Polyethylene glycol (PEG) precipitation of DNA from environmental and faecal samples

The DNA extracted from the environmental samples was further purified by PEG precipitation using a method adapted from Selenska and Klingmuller (1991). An aliquot of 25µl of DNA was transferred to a sterile 1.5ml microcentrifuge tube and 5µl 5M sodium chloride and 25µl 30% PEG 6000 (Sigma-Aldrich Ltd., Gillingham, UK) were added. The sample was mixed and precipitated overnight at 4°C. The sample was then centrifuged at 13,000 ×*g* for 20 minutes and the supernatant removed. The pellet was re-suspended in 1ml of 70% ice-cold ethanol and centrifuged at 13,000 ×*g* for 5 minutes and the supernatant removed. The ethanol wash step was repeated and the resulting pellet was air-dried for at least 30 minutes. The pellet was then re-suspended in 50µl 10mM Tris pH 7.5 and stored at -20°C.

### 2.1.4 DNA quantification

DNA quality and quantity was assessed using nucleic acid purity and yield measurements determined with a Nanodrop® (ND-1000) spectrophotometer (Labtech International Ltd., Luton, UK). Quality was determined based on the A260/280 absorbance ratio. A working detection limit of 3ng $\mu l^{-1}$ was applied to all samples.

## 2.2 Quantitative PCR (qPCR) for *Fusobacterium necrophorum*

### 2.2.1 Details of primers and probe for quantification of *Fusobacterium necrophorum*

A TaqMan® qPCR targeting the *rpoB* gene (RNA polymerase beta subunit) of *F. necrophorum* (Witcomb *et al.*, 2014) was used to analyse all samples (Table 2.1). Primer and probe sets were synthesized and purified commercially (TIB MOLBIOL, GmbH, Berlin, Germany). The probes were labelled at the 5'-end with the fluorescent dye FAM (6-carboxyl-fluorescein) and at the 3'-end with the non-fluorescent quencher BBQ (Black Berry Quencher). Primers were re-suspended in nuclease free water (Ambion®, Life Technologies™, Paisley, UK) to create a stock solution of 100µM (100pmol $\mu l^{-1}$). The stock solution was diluted 1:10 in water to give a 10µM working solution.

**Table 2.1 qPCR primers and TaqMan® probe for quantification of *F. necrophorum* (*rpoB*)**

| | Primer sequence (5' to 3') |
|---|---|
| Forward primer | AACCTCCGGCAGAAGAAAAATT |
| Reverse primer | CGTGAGGCATACGTAGAGAACTGT |
| TaqMan® probe[a] | 6FAM-TCGAACATCTCTCGCTTTTTCCCCGA-BBQ |

[a] 6FAM = 6 carboxy-fluoroscein, BBQ = Black Berry Quencher
(Witcomb *et al.*, 2014)

### 2.2.2 Details of amplification reactions and cycling conditions for the *Fusobacterium necrophorum* qPCR

All qPCR assays were performed using an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Warrington, UK). Cycling conditions and amplification reactions were based on Witcomb *et al*. (2014). PCR mastermix was prepared in a designated hood. The equipment was exposed to UV light for 15 minutes before use. Each sample was run in technical triplicate for quantification purposes; and only samples that were positive for all three technical replicates were used in further analysis; however, where detection rates were predicted to be low, some samples were screened once and then confirmed positives were quantified in triplicate. All reactions were set up in 96 well plates (MicroAmp Fast Optical 96-well Reaction plate, Applied Biosystems, Warrington,

UK). qPCR reactions were carried out in a final volume of 25µl using 2 × TaqMan® Universal Mastermix (Applied Biosystems, Warrington, UK; Table 2.2). DNA from faecal samples was diluted 1:10 before analysis because of the high concentration of DNA in these samples. Reaction conditions consisted of an initial denaturation at 95°C for 20s followed by 40 cycles of 95°C for 30s, 61°C for 30s.

**Table 2.2 Details of master mix components used in qPCR amplifications**

| Master mix component | Working concentration | Final (reaction) concentration | Volume per reaction (µl) |
|---|---|---|---|
| Primer forward | 10µM | 900nM | 2.25 |
| Primer reverse | 10µM | 900nM | 2.25 |
| TaqMan® probe | 10µM | 250nM | 0.625 |
| TaqMan® Universal Mastermix | 2 × | 1 × | 12.5 |
| BSA | 10mg $ml^{-1}$ | 1mg $ml^{-1}$ | 2.5 |
| Nuclease free $H_20$ | - | - | 3.875 |
| DNA | Various | Various | 1.0 |
| Total | - | - | 25.0 |

### 2.2.3 Preparation of a plasmid DNA standard curve for quantification of *Fusobacterium necrophorum*

The *rpoB* gene PCR product from *F. necrophorum* DSM 21784 was purified using the QIAquick Nucleotide Removal kit (Qiagen Ltd., Manchester, UK). The 86bp product was cloned into the pCR® 4-TOPO® vector system (Invitrogen Ltd., Paisley, UK) and the plasmid DNA extracted using the Miniprep kit (Qiagen Ltd., Manchester, UK) as described in Section 2.3. Plasmid DNA was quantified using the Nanodrop® spectrophotometer (Section 2.1.4). Concentrations of DNA required in order to provide serial dilutions of 3.5 × $10^7$ to 3.5 × $10^1$ *rpoB* gene copies $µl^{-1}$ were then calculated as detailed below. The plasmid size including the insert was 4042 bp.

Step 1:

Mass of single plasmid molecule = plasmid size × $1.096 \times 10^{-21}$

Mass of one plasmid molecule: expressed in g
Plasmid size: expressed in bp
$1.096 \times 10^{-21}$: mass of one bp in g

Step 2:

Copy number of interest x mass of single plasmid = mass of plasmid DNA required

Copy number of interest: $3.5 \times 10^{7}$ to 3.5
Mass of plasmid DNA required: expressed in g

Step 3:

Concentration of DNA required = mass of plasmid DNA / volume of DNA added to reaction

Concentration of DNA required: expressed in g $\mu l^{-1}$
Volume of DNA: expressed in µl

### 2.2.4 Analysis of data from the *Fusobacterium necrophorum* qPCR

Results from the qPCR analysis were analysed using the 7500 Fast System SDS Software (Applied Biosystems, Warrington, UK). To maintain consistency in load data from qPCR, results from each 96-well plate were analysed using a set protocol. The threshold value was adjusted to be above all background fluorescence. The standard curve of quantity (*rpoB* gene copies $\mu l^{-1}$) against CT value (number of cycles at which fluorescence exceeds the threshold) was also assessed, and an $R^2$ value ≥ 0.980 was accepted. If $R^2 < 0.980$, the standard furthest from the standard curve was omitted in triplicate. Only results where all three sample triplicates were positive were used in further data analysis.

Results from qPCR analysis were exported to an Excel spreadsheet (2010; Microsoft Corp., Redmond, WA). The mean load of the triplicate run per sample was calculated to determine the number of *rpoB* copies per reaction volume (1µl DNA). This was then multiplied by 50 to determine the number of *rpoB* copies per original sample because DNA was re-suspended in a total volume of 50µl following extraction. The calculation was

then adjusted for any further dilution that had been applied to estimate the number of *rpoB* copies per sample.

### 2.2.5 Analytical specificity and sensitivity of the *Fusobacterium necrophorum* qPCR

The analytical specificity of the *F. necrophorum* (*rpoB*) qPCR has been demonstrated through screening of non-target microorganisms and comparison of sequences from cloned *rpoB* PCR products with the GenBank database (Witcomb *et al.*, 2014). Controls were included to check for contamination during sample processing. A non-template control (nuclease free $H_2O$) was run in triplicate on each 96-well qPCR plate, and extraction blanks (Sections 2.1.1 and 2.1.2) were also analysed using qPCR to ensure no contamination had occurred during the DNA extraction process.

In order to investigate the theoretical detection limit (TDL) for the *F. necrophorum rpoB* qPCR assay, spiking experiments were carried out on swabs, soil and faeces. Sterile swabs (EUROTUBO Collection swab; Delta Lab, Rubi, Spain), 0.5g (+/- 0.05g) soil and 0.1g (+/- 0.01g) faeces (both soil and faeces previously confirmed negative for *F. necrophorum rpoB*) were used for spiking. Cells from a pure culture of *F. necrophorum* (strain DSM 21784; details of culturing method in Section 2.4) were harvested in 2ml sterile PBS and the resulting suspension was used to create ten-fold serial dilutions from undiluted to $10^9$ fold dilution. Spiking samples were inoculated with 100µl (swabs and soil) or 20µl (faeces) of the prepared serial dilutions. Swabs were stored at -80°C in cryotubes (Corning® Cryogenic Vials, Corning Incorporated, New York, USA) containing 300µl sterile PBS, and soil and faeces were stored at -20°C until processing. DNA extraction from the spiked samples was performed as described for each sample type (Section 2.1). A blank sample inoculated with 100µl (swab and soil) or 20µl (faeces) sterile PBS was processed alongside each set of samples. Following extraction, PEG precipitation was performed for soil and faeces samples as described (Section 2.1.3). Samples were analysed in triplicate using the *F. necrophorum rpoB* qPCR assay (Section 2.2.2).

Further 100µl aliquots of each of the dilutions used for spiking were used to provide a reference set for quantification: DNA was extracted from each aliquot using the Qiagen DNeasy Blood and Tissue Kit (Section 2.4.2), and this was then quantified in triplicate using the *F. necrophorum rpoB* qPCR assay (Section 2.2). The results of the qPCR for the spiked samples were compared to this reference set. The lowest quantity detectable for soil and swabs was $10^2$ *rpoB* copies sample$^{-1}$, and for faeces was $10^3$ *rpoB* copies sample$^{-1}$ (Figure 2.1). These values were used as detection limits for subsequent qPCR data.

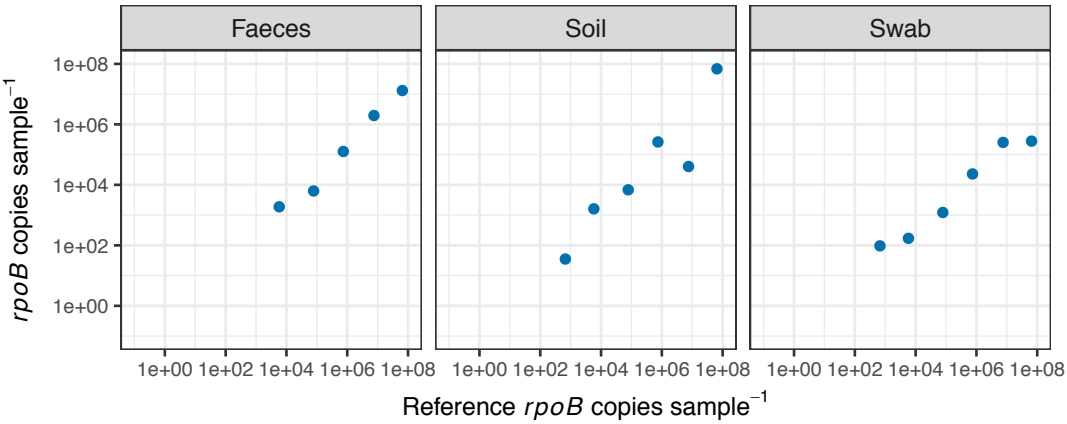


**Figure 2.1 Results of spiking assays**
The number of *rpoB* copies sample$^{-1}$ detected in faeces, soil and swab samples spiked with serial dilutions of *F. necrophorum* are compared to the reference number of *rpoB* copies originally added to those samples.

## 2.3 TOPO TA Cloning® of the *rpoB* gene of *Fusobacterium necrophorum*

### 2.3.1 Method for TOPO TA Cloning®

The TOPO TA Cloning® kit (Invitrogen Ltd., Paisley, UK) was used to clone *rpoB* PCR products from the qPCR assay. Negative control reactions were carried out alongside each cloning reaction. The vector containing the *rpoB* insert was transformed into One Shot® Top 10 competent cells, and ten possible transformants from each sample were selected and incubated overnight at 37°C, in 3ml LB broth containing kanamycin [50µg ml$^{-1}$]. Plasmid DNA was extracted from 1.5ml of this broth using the Miniprep Kit (Qiagen Ltd., Manchester, UK) according to the manufacturer's instructions. DNA was quantified using the Nanodrop® spectrophotometer (Section 2.1.4) and all samples normalised to 15ng µl$^{-}$

[1]. Following PCR with the M13 primer set (Table 2.3), vectors containing the *rpoB* insert produced a single band of 251bp whereas empty vectors produced a single band of 165bp. Products containing the insert were sequenced (Section 2.3.4).

### 2.3.2 Details of PCR used in plasmid vector sequencing

Primers used in PCR reactions for plasmid vector sequencing are presented in Table 2.3. Primers were re-suspended in nuclease free $H_2O$ (Ambion®, Life Technologies™, Paisley, UK) to create stock solutions of 100µM (100pmol $\mu l^{-1}$). The stock solutions were diluted 1:10 in water to give 10µM working solutions.

**Table 2.3 Primers used in PCR reactions for vector sequencing**

| Target | Primer name | Sequence (5' to 3') | Product size (bp) | Reference |
|---|---|---|---|---|
| pCR®4-TOPO plasmid | M13(F)<br>M13(R) | GTAAAACGACGGCCAG<br>CAGGAAACAGCTATGAC | 165 with no insert | Invitrogen Ltd., Paisley, UK |

All PCR reactions were carried out on an Eppendorf Master cycler epgradient machine (Eppendorf, Hamburg, Germany). A positive control (DNA extracted from the type strain *F. necrophorum* DSM 21784) and a blank negative control (nuclease free $H_2O$) were included in all PCR amplifications. PCR reaction mixtures are presented in Table 2.4. EmeraldAmp MAX PCR Master Mix (2 ×) (Takara Bio Europe/SAS, Saint-Germain-en-Laye, France) was used for the M13 vector sequencing PCR. Bovine serum albumin (BSA), (Sigma-Aldrich Ltd., Gillingham, UK), suitable for molecular biology, was also added to PCR reactions as presented in Table 2.4. Cycling parameters were as detailed in the TOPO TA Cloning® kit manual, version N (Invitrogen Ltd., Paisley, UK): 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension period of 72°C for 10 min.

**Table 2.4 PCR reaction mixtures for detection and characterisation of *F. necrophorum* and vector sequencing**

| Master mix component | Working concentration | Final (reaction) concentration | Reaction volume (µl) |
|---|---|---|---|
| Primer forward | 10µM | 400nM | 1 |
| Primer reverse | 10µM | 400nM | 1 |
| EmeraldAmp Mastermix | 2 × | 1 × | 12.5 |
| BSA | 100µg $ml^{-1}$ | 4µg $ml^{-1}$ | 1 |
| Nuclease free $H_20$ | - | - | 8.5 |
| DNA | Various | Various | 1 |
| **Total** | - | - | **25** |

### 2.3.3 Gel electrophoresis of PCR products

PCR products were visualized by ethidium bromide-stained agarose gel electrophoresis using a 1% agarose (Helena Biosciences Europe, Gateshead, UK) gel made with 1 × Tris-acetate-EDTA (TAE) buffer. Each gel was run at 100 volts for 15 - 20 minutes. HyperLadder™ 1kb (Bioline Reagents Ltd., London, UK) was used as the DNA size reference ladder. Gels were visualized under UV light using a Gene Flash imager (Syngene Bio Imaging, Cambridge, UK).

### 2.3.4 Sequencing of PCR products

Sanger sequencing of DNA was carried out using the LIGHTrun sequencing service (GATC Biotech AG, Cologne, Germany). Five µl of purified PCR product at a concentration of 20 - 80ng $µl^{-1}$ were added to 5µl of forward or reverse primer at a concentration of 5µM and submitted for sequencing. Sequences were returned as fasta files and downloaded from a dedicated server at the University of Warwick. Sequence data was compared to the GenBank database using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The search set was defined as the nucleotide collection (nr/nt) and the megablast algorithm (optimised for highly similar sequences) was selected (Morgulis *et al.*, 2008).

## 2.4 Culturing *Fusobacterium necrophorum*

Category II microorganisms were used in this study and relevant safety procedures were followed as appropriate.

### 2.4.1 Anaerobic culturing method for *Fusobacterium necrophorum*

*F. necrophorum* isolates were cultured on a selective media based on that described by Brazier *et al.* (1991). This was Wilkins-Chalgren Anaerobe Agar with the Gram-negative Anaerobe Selective Supplement (Oxoid Ltd., Altrincham, UK), 5% defibrinated sheep blood and josamycin (3µg $ml^{-1}$). They were sub-cultured on Wilkins-Chalgren Anaerobe Agar (Figure 2.2). All incubations were carried out under anaerobic conditions (Don Whitley MACS-MG-1000 anaerobic workstation; 80% $N_2$, 10% $CO_2$ and 10% $H_2$, Don Whitley Scientific Ltd., Shipley, UK) at 30°C for 2-5 days. In some cases where colony identity was uncertain, Gram-staining (Bartholomew & Mittwer, 1952) was used to aid identification of *F. necrophorum* (Figure 2.2; Gram-negative rods, often forming chains). Smears were made by taking material from an isolated colony and spreading onto a microscope slide with 5µl sterile $H_2O$. This was heat fixed before Gram-staining. Cells were viewed using a light microscope under oil using ×100 magnification.

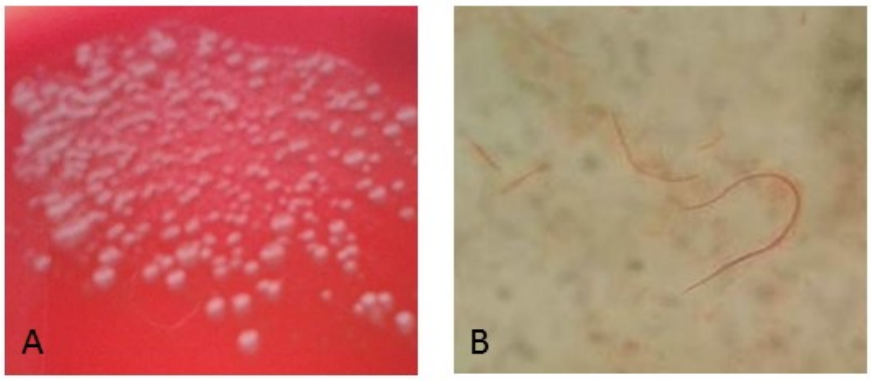


**Figure 2.2 Anaerobic culturing of *F. necrophorum***
A: *F. necrophorum* colonies on Wilkins-Chalgren Anaerobe Agar. B: *F. necrophorum* as viewed under a light microscope with oil using ×100 magnification. Characteristic chains of Gram-negative rods are visible.

### 2.4.2 Confirmation of isolate identity using *Fusobacterium necrophorum* specific PCR

#### 2.4.2.1 Extraction of chromosomal DNA from cultures

Chromosomal DNA was extracted from cultures using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Ltd., Manchester, UK) according to the manufacturer's instructions with a lysis time of 1 hour. Cells were harvested from plates by flooding with 2ml sterile PBS

and scraping with a sterile L-shaped spreader. The resulting suspension was centrifuged and the supernatant discarded. The cell pellet was re-suspended in 180µl ATL buffer solution provided in the kit and this suspension used for extraction. At the final stage of the extraction two DNA elutions were performed to increase DNA yield as suggested by the manufacturer, each in 150µl of elution buffer. Both aliquots of DNA were then stored at -20°C.

#### 2.4.2.2 *Fusobacterium necrophorum* specific PCR of DNA extracted from cultures

DNA extracted from cultures was tested using PCR to confirm that the cultured organism was *F. necrophorum*. Primers synthesised by Sigma-Aldrich (Sigma-Aldrich Ltd., Gillingham, UK) targeting the gyrase β subunit of *F. necrophorum* (Jensen *et al.*, 2007) and the haemagglutinin of *F. necrophorum* subsp. *necrophorum* (Narongwanichgarn *et al.*, 2003) were used to detect and characterise *F. necrophorum* isolates (Table 2.5).

**Table 2.5 Primers used in detection and characterisation of *F. necrophorum* isolates**

| Target | Primer name | Sequence (5' to 3') | Product size (bp) |
|---|---|---|---|
| *F. necrophorum* (gyrase β subunit) | GyrB (F)<br>GyrB (R) | AGGATTGCATGGAGTAGGAA<br>CCTATTTCATTTCGACAATCCA | 306 |
| *F. necrophorum* subsp. *necrophorum* (haemagglutinin) | Wlf2<br>Wlr1 | AGGTGCTTCTTCCACAGC<br>GCACCATTTTGAGCGCGT | 250 |

PCR reactions were carried out as described for vector sequencing PCR reactions in Section 2.3.2, with the exception of the cycling conditions which were 95°C for 5 min followed by 32 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, with a final extension period of 72°C for 10 min (Antiabong *et al.*, 2013a). Gel electrophoresis was carried out as described in Section 2.3.3.

# Chapter 3 Understanding persistence of *Fusobacterium necrophorum* in sheep and their environment: two longitudinal studies

## 3.1 Introduction

*Fusobacterium necrophorum* is an opportunistic, secondary pathogen that increases the severity of ovine footrot (Beveridge, 1941; Roberts & Egerton, 1969; Witcomb *et al.*, 2014). Opportunistic pathogens have a reservoir in healthy individuals or their environment, and knowledge of these reservoirs improves our understanding of disease epidemiology, pathogenesis and control (Anttila *et al.*, 2015).

Our understanding of the reservoirs of *F. necrophorum* in footrot is poor. The literature states that the environment and sheep faeces are the primary reservoirs for *F. necrophorum* in footrot (Roberts & Egerton, 1969; Langworth, 1977), however, in a recent study *F. necrophorum* was not detected in soil from sheep pasture or sheep faeces (Witcomb, 2012). *F. necrophorum* can be detected on the healthy feet and in the mouths of sheep (McCourtie *et al.*, 1990; Witcomb, 2012; Witcomb *et al.*, 2014; Frosth *et al.*, 2015; Maboni *et al.*, 2016), but these data are mainly cross sectional. There have been no longitudinal studies on persistence of *F. necrophorum* at any of these sites under natural conditions.

The role of the environment in transmission of *F. necrophorum* has been observed in deer and cattle (Monrad *et al.*, 1983; Edwards *et al.*, 2001), but only under conditions of high stocking density and high rainfall. Contamination of the feet of sheep with faeces results in colonisation with *F. necrophorum* (Roberts & Egerton, 1969), but again this was demonstrated in wet conditions and at high stocking density. There are no observational studies that link *F. necrophorum* presence at reservoir sites to footrot in sheep kept at pasture.

The aim of this study was to determine sites of persistence of *F. necrophorum* in sheep and their environment using longitudinal data on *F. necrophorum* presence and load in samples collected from feet, mouths, and faeces of sheep, as well as soil and grass from sheep pasture. These findings were compared for high and low transmission situations (Study A and B respectively). Additionally, the aim was to investigate whether changes in *F. necrophorum* presence and load at these sites were associated with changes in incidence, prevalence, severity and chronicity of footrot.

## 3.2 Materials and Methods

### 3.2.1 Approvals and consent

The studies were approved by the University of Warwick's local ethics committee (AWERB.33/13-14). Faecal samples from sheep were collected under Home Office Licence (PPL 70/8392). Informed consent was obtained from the farmers before each study started and farmers were compensated for inconvenience at the end of the studies. The farmers were notified of any footrot lesion score > 1, and treatment was advised. All treatments were recorded.

### 3.2.2 Study design and sheep sampling procedure

Two longitudinal studies (Studies A and B) were carried out. There were 10 sheep in Study A and 40 sheep in Study B. The data from these studies were also used to study *Dichelobacter nodosus;* load of *D. nodosus* is included in the statistical analyses in this current chapter.

Study A was carried out as a pilot study, and therefore included a small number of sheep over a short time period. The aim of this study was to gain initial information regarding the sites where *F. necrophorum* was present and persisted, and to inform the design of Study B. The choice of 10 sheep over 4 visits was based on having sufficient information to achieve these aims whilst fitting into constraints on time and budget. Both healthy and diseased sheep were selected to ensure that all disease states were represented in the pilot data.

Study B was carried out over a 5 month period in order to cover the changing climatic conditions from winter into early summer. It was decided to use a group of healthy sheep and to move them to an un-grazed pasture so that the full disease progression could be followed for all sheep. A sample size calculation was carried out to determine the number of sheep required (Petrie & Watson, 2013), however, it was difficult to determine an accurate number due to the lack of previous evidence regarding detection rates for faecal

samples and mouth swabs from sheep in different disease states. Based on the available evidence for foot swabs and considering different degrees of correlation, a range of 7 – 25 sheep was needed to detect a difference in detection frequency between disease states. A range of 6 – 44 sheep was needed to detect a $\log_{10}$ increase in *F. necrophorum* load, again considering different degrees of correlation. Practical considerations regarding the number of sheep that could be sampled within one day were also taken into account, and therefore it was decided to sample 40 sheep. It was decided to use a sampling interval of 1 week based on results from Study A and Witcomb *et al.* (2014).

#### 3.2.2.1 Study design and sheep sampling for Study A

The study population was a flock of approximately 150 North Country Mule breeding ewes on a lowland farm in Warwickshire, England where footrot was endemic. The flock was first visited on 28-May-2014. Four lame sheep (two ewes and two lambs) were convenience-selected and six non-lame sheep were randomly selected. These 10 sheep were sampled fortnightly for four occasions. They remained as part of the larger flock throughout the study period and grazed only one pasture. At the final visit five ewes and three lambs were sampled, because two lambs had been sold.

At each sampling, each foot of each sheep was examined and scored for lesions of interdigital dermatitis (ID) and severe footrot (SFR) as described by Moore et al. 2005 (Table 3.1). Body condition score was assessed for each sheep at each visit (Defra, 2011). Data were recorded on paper recording sheets.

**Table 3.1 Scoring system for lesions of interdigital dermatitis (ID) and severe footrot (SFR) from Moore et al. 2005**

| Lesion score[a] | Description |
|---|---|
| *Interdigital dermatitis* | |
| 0 | Clean interdigital foot with no dermatitis (scald) lesion or fetid smell |
| 1 | Slight interdigital dermatitis, irritation of the skin but dry |
| 2 | Slight interdigital dermatitis with a fetid smell, < 5% skin affected |
| 3 | Moderate interdigital dermatitis with a fetid smell, 5-25% skin affected |
| 4 | Severe interdigital dermatitis with a fetid smell, > 25% skin affected |
| *Severe footrot* | |
| 0 | A clean digit with no lesion |
| 1 | An active or healing footrot lesion with a degree of separation of the sole |
| 2 | An active footrot lesion with a marked degree of separation of the sole |
| 3 | An active footrot lesion with extensive under-running of the wall hoof horn (may include under-running of the sole) |
| 4 | An active footrot lesion with complete under-running of the wall hoof horn (may include under-running of the sole) |

[a] One score for each lesion was recorded per foot

The interdigital skin of each foot was sampled (Figure 3.1) using a sterile cotton swab (EUROTUBO Collection swab; Delta Lab, Rubi, Spain). The swab was swiped 5 times from proximal to distal across the skin. After the feet were sampled the gingival crevice (gum-tooth margin) of the lower incisors was sampled (Figure 3.1) using a sterile cotton swab. Gloves were worn for sample collection and were changed between sheep. The samples collected are summarised in Table 3.2.


**Figure 3.1 Sheep sample collection**
A: Sheep turned into a sitting position for sampling. B: Taking a swab sample from the interdigital skin. C: Taking a swab sample from the gingival crevice

**Table 3.2 Summary of samples collected from sheep in the two longitudinal studies**

| Sample type | No. per visit | | Samples collected per sampling episode |
|---|---|---|---|
| | Study A | Study B | |
| Foot swabs | 40 | 160 | One swab sample per foot |
| Mouth swabs | 10 | 40 | One swab sample from gingival crevice per sheep |
| Faeces | NA | 40 | Study B only: one rectal faecal sample per sheep |

### 3.2.2.2 Study design and sheep sampling for Study B

The study population was 120 Suffolk cross Wiltshire Horn ewe lambs on a farm in Warwickshire, England. The flock was chosen based on a known history of footrot.

On 07-Feb-2015 baseline samples from the study pasture were taken, and the pasture was left empty until sampled again on 17-Feb-2015.

On 18-Feb-2015, a study group of 40 individuals was selected from a group of 120 ewe lambs (1 year old females that had not been bred). All 120 sheep were observed for lameness and divided into three groups: non-lame, lame and those where lameness was uncertain. The non-lame sheep were examined and lesion scored as described for Study A (Section 3.2.2.1). Data were recorded using an electronic ID (EID) reader (Agrident APR500) with custom-designed software (Border Software Ltd., UK). Forty healthy sheep (non-lame, no SFR lesion, ID lesion scored ≤ 1) were identified and samples collected as described for Study A except a rectal faecal sample was also collected from each sheep using a clean, gloved finger. If insufficient faecal material was present, a rectal swab was taken.

These 40 animals formed the study group for the longitudinal study, and were moved to the study pasture. The study sheep and pasture were sampled every week from 25-Feb-2015 to 01-Jul-2015. There were total of 20 sampling occasions including the samples taken on 18-Feb-2015.

### 3.2.3 Procedure for collecting environmental samples for the studies

#### 3.2.3.1 Identification of pasture sites

Soil and grass samples were collected from the pasture grazed by the study group. Two high traffic areas and one low traffic area were sampled (Table 3.3). High traffic areas were those where sheep congregated or visited more frequently based on observation of the sheep and information from the farmer. Low traffic areas were those where sheep did not congregate or visit frequently.

**Table 3.3 Details of high and low traffic areas for Studies A and B**

| Location | Details | Samples Type | Depth [a] | No. per visit |
|---|---|---|---|---|
| *Study A* | | | | |
| High traffic 1 | Large tree used for shelter | Soil | 0-1cm | 3 |
| | | | 4-5cm | 3 |
| | | Grass | --- | 1[b] |
| High traffic 2 | Open gateway to adjacent field | Soil | 0-1cm | 3 |
| | | | 4-5cm | 3 |
| | | Grass | --- | 0[b] |
| Low traffic | 20m × 20m area | Soil | 0-1cm | 5 |
| | | | 4-5cm | 5 |
| | | Grass | --- | 5 |
| *Study B* | | | | |
| High traffic 1 | Ring feeder | Soil | 0-1cm | 3 |
| | | | 4-5cm | 3 |
| | | Grass | --- | 2-3[b] |
| High traffic 2 | Water trough by hedge | Soil | 0-1cm | 3 |
| | | | 4-5cm | 3 |
| | | Grass | --- | 1-3[b] |
| Low traffic | 20m × 20m area | Soil | 0-1cm | 5 |
| | | | 4-5cm | 5 |
| | | Grass | --- | 5 |

[a] Depth from which soil sample taken relative to surface of pasture
[b] Grass collected where present

### 3.2.3.2 Sampling procedure for pasture sites

In low traffic areas a 20m × 20m area was divided into 5m × 5m squares (Figure 3.2) with nodes on the quadrant numbered 1 - 25 and five chosen each week using a random number generator (https://www.randomizer.org). In high traffic areas, a sample was taken from the centre of each area and at 1m and 2m distant (Figure 3.2).

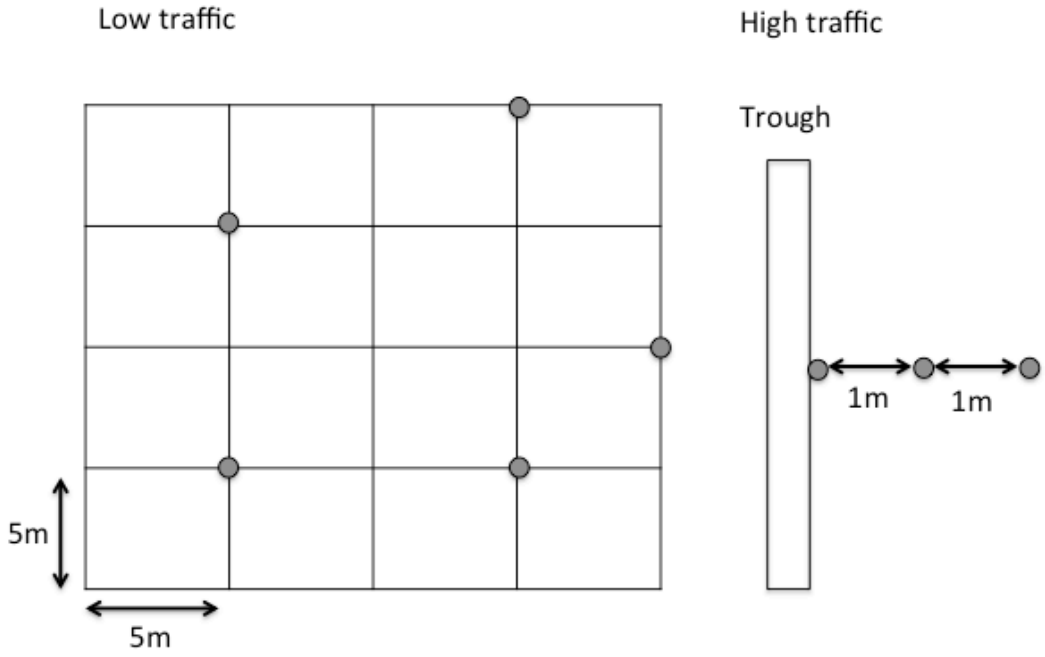


**Figure 3.2 Sampling strategy for low and high traffic areas of pasture**
Sampling points are shown using grey circles. For the low traffic quadrant, nodes are numbered from 1 – 25 and five were selected using a random number generator. For the high traffic area, a sample was taken at the site and at 1m and 2m distant.

Soil samples were collected using a soil corer (diameter 3.5cm). Sections of soil at 4-5cm and 0-1cm depth (relative to the soil surface) from each sampling point were transferred to sterile universal containers using spatulas cleaned with DNA remover™ wipes (Minerva Biolabs GmbH, Berlin, Germany) between samples. Grass samples were picked and transferred to sterile universal containers using disposable gloves that were changed between samples.

#### 3.2.3.3 Collection of faecal samples from pasture (Study A only)

In Study A, faecal samples were collected from pasture. Five fresh faecal samples were collected from five sites on the pasture using clean gloves that were changed between sample collections. External and internal (i.e. no direct contact with the environment) sections of each faecal sample were collected, giving ten samples per visit.

### 3.2.4 Sample storage

Swab samples were placed in 2 ml sterile cryotubes (Corning® Cryogenic Vials, Corning Incorporated, New York, USA) containing 300µl sterile phosphate buffered saline (PBS) and transported to the university on ice before being stored at -80°C. All environmental and faecal samples were placed in labelled 25ml sterile universal containers (SARSTEDT AG & Co., Nümbrecht, Germany) and frozen at -20°C on return to the University.

### 3.2.5 Collection of climate data

#### 3.2.5.1 Collection of weather data for Studies A and B

Daily data on total rainfall (mm) and minimum, maximum and mean temperatures (°C) were accessed from the Warwick weather station (http://warwick-weather.com/; last accessed Aug 2015, no longer available). Data were recorded from ten days before the start of each study until the end of the study.

#### 3.2.5.2 Collection of soil temperature and moisture data (Study B only)

Soil temperature and moisture were collected from the high and low traffic areas. Soil temperature was recorded using a spirit thermometer at 1cm depth (Fisherbrand™, Fisher Scientific UK Ltd., Loughborough, UK). Two soil samples per area one from 0-1 cm depth and one from 4-5 cm depth were collected to determine soil moisture. These samples were weighed, and then dried at 110°C for 24 hours and then weighed again. Soil moisture was calculated using the formula:

$$MC\% = ((W_2 - W_3)/(W_3 - W_1)) \times 100$$

MC% = soil moisture content as a percentage
$W_1$ = weight of soil container (g)
$W_2$ = weight of moist soil with container (g)
$W_3$ = weight of dry soil with container (g)

### 3.2.6 Laboratory analysis of samples collected during longitudinal studies

DNA was extracted from samples collected from sheep and their pasture as described in Section 2.1. Load of *F. necrophorum* was measured in these samples using qPCR as

described in Section 2.2. All samples from Study A were analysed. All environmental samples (soil and grass) from Study B were analysed plus a subset of sheep samples. This included samples from 19 diseased sheep from 2 weeks before a period of footrot (or start of the study) to 2 weeks after the period of footrot (or end of the study). Samples from every fourth week from 2 sheep that scored ID0 and SFR0 for the duration of the study were analysed. Samples from weeks 1-3 were analysed for these 21 sheep, plus a further randomly selected 9 sheep. The samples selected are shown in Appendix 3.

### 3.2.7 Data preparation

#### 3.2.7.1 Data preparation for Study A

Sheep and climate data were manually entered into an Excel spreadsheet (2010; Microsoft Corp., Redmond, WA) together with qPCR data on load of *F. necrophorum* and *D. nodosus* (Section 2.2.4).

#### 3.2.7.2 Data preparation for Study B

Sheep data were downloaded each week from the EID reader into text files and imported into the R statistical environment (R Development Core Team, 2008) and combined into one data file using the reshape2 package[1] (Wickham, 2007). qPCR data on load of *D. nodosus* and *F. necrophorum* (Section 2.2.4) and environmental data were manually entered into an Excel spreadsheet and then imported into R and merged with sheep data.

### 3.2.8 Statistical analysis of qPCR data from longitudinal studies

All statistical analyses were carried out using the R (v3.3.2) statistical environment (R Development Core Team, 2008) with the R studio user interface (v1.0.136). (Bacterial load data + 1) were $\log_{10}$ transformed for statistical analyses. For the purpose of statistical analysis, footrot status was defined as presented in Table 3.4. Feet could be classed as healthy or having footrot, and footrot could be further categorised as ID or SFR. The

[1] Dr R.E. Crump is acknowledged for writing the code for importing and combining original sheep data files.

footrot status of a sheep was determined in the same way based on the most severe lesion recorded on her feet at that time.

**Table 3.4 Classification of footrot status for statistical analysis**

| Term | Lesion score |
|---|---|
| Healthy | ID ≤ 1, SFR 0 |
| Footrot (includes ID and SFR) | ID > 1 and/or SFR > 0 |
| ID | ID > 1 and SFR 0 |
| SFR | SFR > 0 |

#### 3.2.8.1 Chi-squared test for goodness of fit

Expected frequencies for number of positive samples by sample type were calculated by multiplying the overall detection rate by the total number of samples of each type collected. A Chi-squared test was then used to determine goodness of fit. P-values of ≤ 0.05 were considered significant.

#### 3.2.8.2 Associations between footrot status and load of *Fusobacterium necrophorum* on foot swabs, mouth swabs and in faecal samples

Due to the presence of repeated measures in the data, two level binomial mixed effects models were used to determine associations between footrot status and load of *F. necrophorum* on positive foot swabs, mouth swabs, and faecal samples (Study B only). The outcome variable was the presence/absence of footrot, and data were grouped by foot (foot swabs) or sheep (mouth swabs and faecal samples) to account for repeated observations. The $\log_{10}$ transformed load of *F. necrophorum* was used as the explanatory variable. Models were constructed using the "glmer" function from the lme4 package in R (Bates *et al.*, 2015) with the "bobyqa" optimizer and $1 \times 10^5$ as the maximum number of function evaluations. Associations between load and footrot status were considered significant when 95% confidence intervals of the coefficient for load did not include 0.

#### 3.2.8.3 Survival analysis

Non-parametric maximum likelihood estimation (Kaplan-Meier estimate) of survival of *F. necrophorum* positive samples was carried out using the "icfit" function from the interval

package (Fay & Shaw, 2010). The event was a sample becoming negative for *F. necrophorum*. Data were interval censored: the time period was grouped into two weekly intervals for Study A, and weekly intervals for Study B, with events occurring during these intervals but exact time of events being unknown. Assessment times were independent of event times i.e. sampling times were predetermined rather than depending on whether an event had occurred. The "ictest" function from the interval package was used to carry out the Wilcoxon two sample permutation test for differences in survival probabilities between groups. The "ggplot" function from the ggplot2 package (Wickham, 2009) was used to plot survival probabilities.

#### 3.2.8.4 Binomial mixed effects models for presence of *Fusobacterium necrophorum* on foot swabs and mouth swabs

Data from each study were analysed using binomial logistic mixed effects models. Separate models were constructed for the outcomes for feet and mouths. A three-level model was used when presence/absence of *F. necrophorum* on feet was the outcome to account for repeated observations over time of feet and feet clustered within sheep. A two-level model was used when presence/absence of *F. necrophorum* in mouths was the outcome to account for repeated observations over time of the mouth of each sheep. Models were constructed using the "glmer" function as described in Section 3.2.8.2. For the model of foot swab data from Study B, a mean-centred polynomial term for time (week + week$^2$ + week$^3$ + week$^4$) was included in all analyses. Explanatory variables were lagged to the previous time point (2 weeks for Study A, 1 week for Study B). Explanatory variables were initially tested individually in univariable models and then a multivariable model was developed using a manual forward selection process. The Akaike information criterion (AIC) was used to compare the relative fit of models. Variables were retained in the model when 95% confidence intervals of the coefficient did not include 0, and when the AIC value for the model was lower. Where variables could not be assessed in the binomial model due to lack of sufficient data to determine model parameters, Fisher's Exact test was used to test for an association between presence of *F. necrophorum* and the lagged explanatory variable.

#### 3.2.8.5 Linear mixed effects models for load of *Fusobacterium necrophorum* on foot swabs (Study B only)

A linear mixed effects model was constructed with $\log_{10}$ *F. necrophorum* load data from positive foot swabs from Study B as the outcome variable. A three-level model was used as for the binomial model for foot swabs above. Models were constructed using the "lme" function from the nlme package (Pinheiro *et al.*, 2016). Lagged explanatory variables were tested in univariable and multivariable models as described above.

#### 3.2.8.6 Associations and correlations between explanatory variables

Associations between continuous and ordinal explanatory variables were examined using Spearman's correlation tests. Associations between binary categorical variables were examined using a Chi-squared test, and associations between a binary categorical variable and a continuous or ordinal variable were examined using a Mann Whitney U test.

## 3.3 Results for Study A

### 3.3.1 Prevalence of footrot during Study A

There were 152 foot observations over the course of the study. Lesion scores observed were 0 – 4 for ID and 0 – 3 for SFR. Only one foot had footrot for more than 2 weeks. Prevalence of footrot increased during the course of the study with highest prevalence in week 5 (Figure 3.3). One ewe and one lamb were healthy for the entire study period. Individual lesion scores and treatments are presented in Appendix 1.

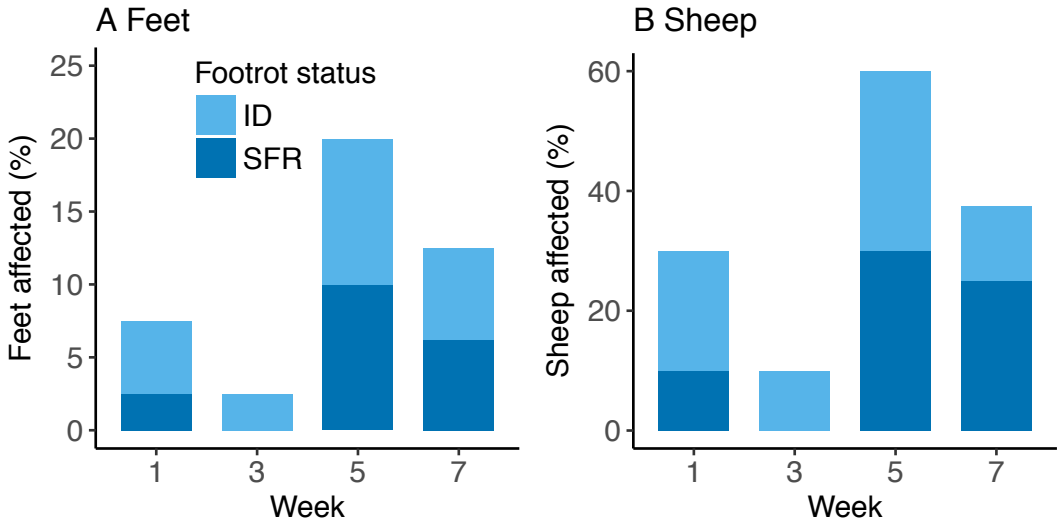


**Figure 3.3 Footrot prevalence during Study A**
A: percentage of feet affected, B: percentage of sheep affected.

### 3.3.2 Climate data from Study A

Climate data are presented in Figure 3.4. Mean temperature ranged from 12.2°C to 17.6°C. Rainfall was high from 10 days before the first visit to the second visit and it then became drier, with one two-week period with less than 1mm rainfall between the second and third visits.

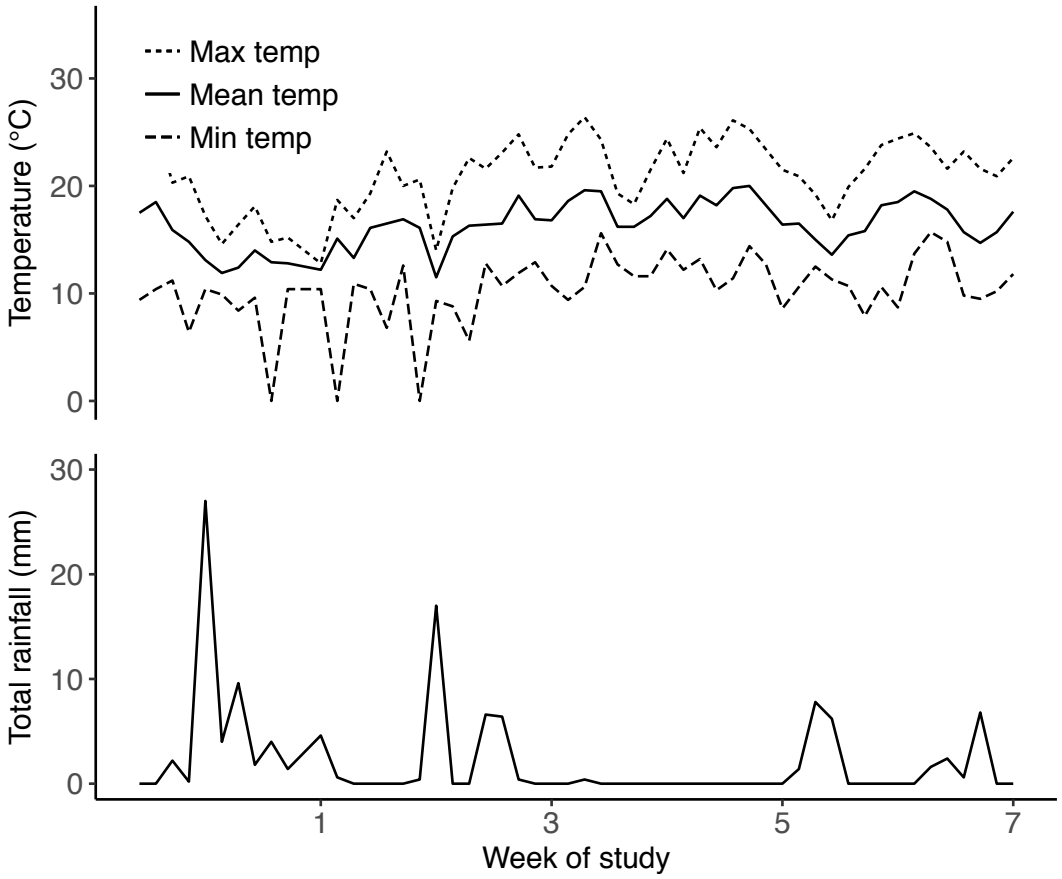


**Figure 3.4 Climate data for Study A**
Data from ten days prior to commencement of the study to the end of the study (week 7) are shown. Upper panel: max (maximum), mean and min (minimum) daily temperature in °C. Lower panel: daily rainfall in mm.

### 3.3.3 Detection and quantification of *Fusobacterium necrophorum* for Study A

#### 3.3.3.1 Detection of *Fusobacterium necrophorum* by sample type

The overall detection rate of *F. necrophorum* in samples from Study A was 34%. *F. necrophorum* was detected in 50% of foot and 78.9% of mouth swabs and rarely detected in the environment or faecal samples (Table 3.5). There were more positive foot and mouth samples, and fewer positive environmental and faecal samples than expected by chance ($p < 0.01$).

**Table 3.5 Frequency of detection and load of *F. necrophorum* by sample type for Study A**

| Sample type | Frequency of detection | | *rpoB* copies in positive samples | |
|---|---|---|---|---|
| | No. | % | Minimum | Maximum |
| Foot swabs | 76/152 | 50.0 | $1.42 \times 10^2$ swab$^{-1}$ | $8.37 \times 10^7$ swab$^{-1}$ |
| Mouth swabs | 30/38 | 78.9 | $1.16 \times 10^2$ swab$^{-1}$ | $1.08 \times 10^6$ swab$^{-1}$ |
| Faeces | 1/40 | 2.5 | $7.27 \times 10^6$ g$^{-1}$ | $7.27 \times 10^6$ g$^{-1}$ |
| Soil | 7/88 | 8.0 | $3.24 \times 10^3$ g$^{-1}$ | $1.02 \times 10^5$ g$^{-1}$ |
| Grass | 1/24 | 4.2 | $1.71 \times 10^4$ g$^{-1}$ | $1.71 \times 10^4$ g$^{-1}$ |

### 3.3.3.2 Variation in detection and load of *Fusobacterium necrophorum* with footrot status

Detection rates of *F. necrophorum* on foot swabs and mouth swabs were highest in feet and sheep with footrot (Table 3.6). Foot swabs with higher loads of *F. necrophorum* were more likely to be from feet with ID or SFR than foot swabs with lower loads (OR 3.64, 95% CI 2.04-8.18; Figure 3.5). Load of *F. necrophorum* on mouth swabs was not associated with footrot status (Figure 3.5).

**Table 3.6 Detection of *F. necrophorum* on foot swabs and mouth swabs by footrot status for Study A**

| | Healthy | | ID | | SFR | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| Foot swabs | | | | | | |
| *Sheep level* | 52/100 | 52 | 9/28 | 32.1 | 15/24 | 62.5 |
| *Foot level* | 67/136 | 49.3 | 3/9 | 33.3 | 6/7 | 85.7 |
| Mouth swabs | 20/25 | 80 | 4/7 | 57.1 | 6/6 | 100 |

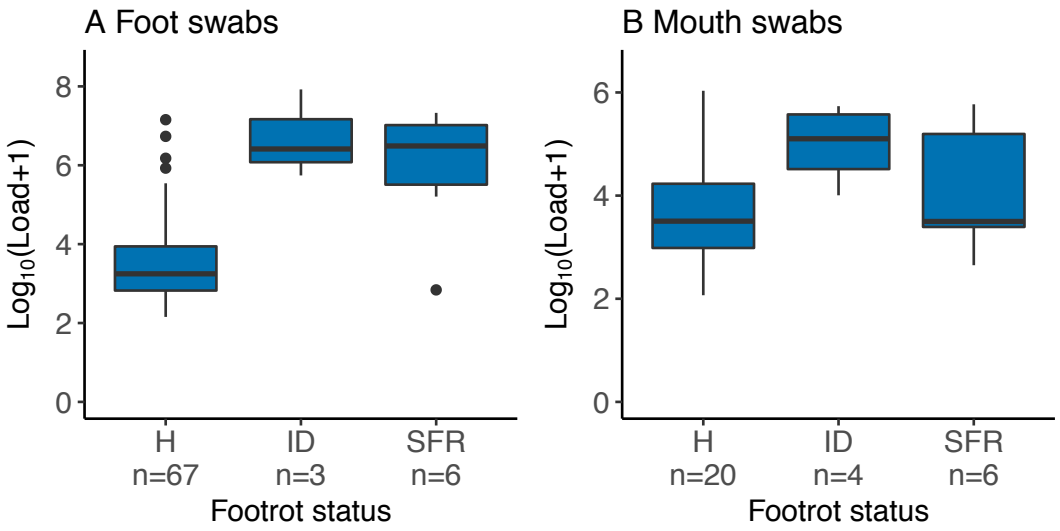


**Figure 3.5 Load of *F. necrophorum* on foot and mouth swabs by footrot status for Study A**

A: foot swabs and B: mouth swabs.

#### 3.3.3.3 Variation in detection and load of *Fusobacterium necrophorum* over time

Detection of *F. necrophorum* on foot swabs and in environmental samples (soil and grass) decreased over the course of the study (Table 3.7). Only one faecal sample was positive for *F. necrophorum* (week 3). Detection in mouth samples was high throughout the study (60-90% of swabs positive per visit).

**Table 3.7 Detection of *F. necrophorum* in all sample types by week for Study A**

| | Samples with detectable *F. necrophorum* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Week | Foot swabs | | Mouth swabs | | Soil | | Grass | | Faeces | |
| | No. | % | No. | % | No. | % | No. | % | No. | % |
| 1 | 39/40 | 97.5 | 9/10 | 90.0 | 6/22 | 27.3 | 0/6 | 0 | 0/10 | 0 |
| 3 | 17/40 | 42.5 | 9/10 | 90.0 | 1/22 | 4.5 | 1/6 | 16.7 | 1/10 | 10.0 |
| 5 | 11/40 | 27.5 | 6/10 | 60.0 | 0/22 | 0 | 0/6 | 0 | 0/10 | 0 |
| 7 | 9/32 | 28.1 | 6/8 | 75.0 | 0/22 | 0 | 0/6 | 0 | 0/10 | 0 |

#### 3.3.3.4 Variation in detection of *Fusobacterium necrophorum* between environmental sampling locations

There were few positive soil samples. The majority (6/7) of positive soil samples were from surface soil, and 6/7 were from high traffic areas (Table 3.8). The one positive grass sample was from a low traffic area.

**Table 3.8 Detection of *F. necrophorum* at different pasture sampling sites for Study A**

| Sample type and location | Positive samples | |
|---|---|---|
| | No. | % |
| Soil | | |
| *High traffic* | 6/48 | 12.5 |
| *Low traffic* | 1/40 | 2.5 |
| | | |
| *Surface (0-1cm)* | 6/44 | 13.6 |
| *Deep (4-5cm)* | 1/44 | 2.3 |
| | | |
| Grass | | |
| *High traffic* | 0/4 | 0 |
| *Low traffic* | 1/20 | 5 |

### 3.3.3.5 Persistence of *Fusobacterium necrophorum* over time

From survival analysis, mouth samples were more likely to remain positive throughout the study than foot swabs or environmental samples ($p < 0.01$; Figure 3.6). All environmental samples were negative for *F. necrophorum* by week 5 and only 8% of foot samples that were positive at the start of the study remained positive throughout the study period.

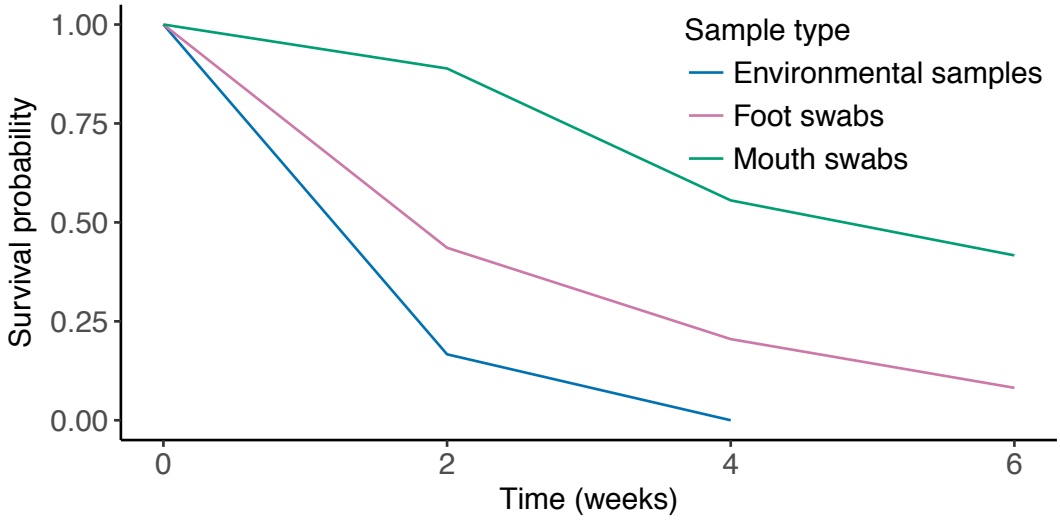


**Figure 3.6 Survival probability by sample type for Study A**
The probability of samples positive at the start of the study remaining positive over time is plotted for foot swabs, mouth swabs and environmental samples.

### 3.3.4 Binomial mixed effects models of presence of *Fusobacterium necrophorum* on foot swabs

The results for the univariable analysis are shown in Table 3.9.

**Table 3.9 Univariable binomial mixed effects regression model of presence of *F. necrophorum* on foot swabs as determined by qPCR for Study A**

| Value two-weeks previously | No. | % | Odds ratio | Lower 95% CI | Upper 95% CI | P value < 0.2 |
|---|---|---|---|---|---|---|
| *Foot level* | | | | | | |
| $Log_{10}$(Fn[a] load + 1) | 112 | 100 | 1.45 | 1.16 | 1.82 | * |
| $Log_{10}$(Dn[a] load + 1) | 112 | 100 | 1.35 | 1.00 | 1.93 | * |
| Foot with footrot | 9 | 8 | 14.4 | 2.31 | 154 | * |
| Antibiotic spray treatment | 21 | 19 | 3.19 | 0.88 | 12.1 | * |
| Foot trimming | 4 | 4 | 4.06 | 0.26 | 74.4 | |
| *Sheep level* | | | | | | |
| Mouth swab Fn positive | 92 | 82 | 6.05 | 1.27 | 41.7 | * |
| Sheep with footrot | 32 | 29 | 4.44 | 1.29 | 19.4 | * |
| Sheep positive on at least one foot | 96 | 86 | 0.86 | 0.10 | 6.10 | |
| Lamb versus ewe | 52 | 46 | 3.29 | 0.64 | 27.2 | * |
| *Time* | | | | | | |
| Week 3 | 40 | 36 | Ref | | | |
| Week 5 | 40 | 36 | 0.39 | 0.11 | 1.18 | * |
| Week 7 | 32 | 29 | 0.51 | 0.15 | 1.66 | * |

Variables with p < 0.2 included in subsequent multivariable analysis are indicated by *. No. and % refer to the number and percentage of values in the dataset. CI = confidence interval. Ref = baseline category for comparison.
[a] Fn refers to *F. necrophorum* and Dn refers to *D. nodosus*

Two explanatory variables remained in the multivariable model (Table 3.10). The likelihood of a foot being positive for *F. necrophorum* increased as the log load of *F. necrophorum* on that foot two weeks previously increased (OR 1.47, 95% CI 1.16-1.89). A sheep having at least one foot with footrot was associated with increased odds of a foot being positive for *F. necrophorum* two weeks later compared to a sheep without footrot on any feet (OR 3.40, 95% CI 1.11-12.7).

**Table 3.10 Multivariable binomial mixed effects regression model of presence of *F. necrophorum* on foot swabs as determined by qPCR for Study A**

| Value two-weeks previously | No. | % | Odds ratio | Lower 95% CI | Upper 95% CI |
|---|---|---|---|---|---|
| *Fixed effects* | | | | | |
| $Log_{10}$ (Fn[a] load + 1) foot | 112 | 100 | **1.47** | 1.16 | 1.89 |
| Sheep healthy | 80 | 71.4 | Ref | | |
| Sheep with footrot | 32 | 28.6 | **3.40** | 1.11 | 12.7 |
| *Random part* | | | | | |
| Variance (foot level) | 0.00 | | | | |
| Variance (sheep level) | 0.76 | | | | |

No. and % refer to the number and percentage of values in the dataset. CI = confidence interval. Ref = baseline category for comparison. Where odds ratios are in bold, they are statistically significant at 0.05 when CI do not include unity.
[a] Fn refers to *F. necrophorum*

#### 3.3.4.1 Correlations and associations between explanatory variables

Associations between explanatory variables in the binomial model of foot swab data are shown in Appendix 2. There was a positive correlation between load of *F. necrophorum* and a foot having footrot, but not with load of *D. nodosus*. Lambs were more likely to have higher loads of *F. necrophorum* than ewes. There was a negative correlation between *F. necrophorum* load and time. A sheep having footrot was associated with antibiotic spray treatment.

### 3.3.5 Binomial mixed effects models of presence of *Fusobacterium necrophorum* on mouth swabs

The results for the univariable mouth swab analysis are shown in Table 3.11. Footrot status at the previous time point could not be analysed in the model as there were no instances where a sheep had footrot at the previous time point and had a negative mouth swab for *F. necrophorum* so parameters could not be uniquely determined. From analysis using Fisher's exact test, sheep with footrot at the previous time point were more likely to have a positive mouth swab (p = 0.07). Presence (and load) of *D. nodosus* in the mouth at the previous time point could not be analysed in the model as parameters could not be

uniquely determined. From analysis using Fisher's exact test, there was no association between presence of *D. nodosus* in the mouth at the previous time point and presence of *F. necrophorum* in the mouth. The likelihood of a positive mouth swab was not associated with any of the variables tested in the model, but the small number of data points (n=28) may have been a contributing factor.

**Table 3.11 Univariable binomial mixed effects regression model of presence of *F. necrophorum* on mouth swabs as determined by qPCR for Study A**

| Value two-weeks previously | No. | % | Odds ratio | Lower 95% CI | Upper 95% CI |
|---|---|---|---|---|---|
| $Log_{10}$ (Fn[a] load + 1) on mouth swab | 28 | 100 | 1.39 | 0.81 | 2.34 |
| Sheep positive on at least one foot | 24 | 85.7 | 0.90 | 0.03 | 11.1 |
| Lamb versus ewe | 13 | 46.4 | 0.55 | 0.03 | 5.25 |
| | | | | | |
| *Time* | | | | | |
| Week 3 | 10 | 35.7 | Ref | | |
| Week 5 | 10 | 35.7 | 0.13 | 0.00 | 1.38 |
| Week 7 | 8 | 28.6 | 0.30 | 0.01 | 4.36 |

No. and % refer to the number and percentage of values in the dataset.
CI = confidence interval. Ref = baseline category for comparison.
[a] Fn refers to *F. necrophorum*

## 3.4 Results from Study B

### 3.4.1 Prevalence of footrot during Study B

There were 3192 foot observations during the study. There were two missing data points: sheep 03499 on weeks 5 and 8. The scores for both ID and SFR ranged from 0 – 3, and the duration of footrot (number of consecutive weeks with lesions recorded) ranged from 1 to 8 weeks. The prevalence of footrot peaked in week 5, and there was no footrot recorded during week 11 (Figure 3.7). Ten sheep remained healthy over the course of the study. Only one sheep received treatment during the study period (antibiotic injection given to sheep 03535 in week 9). Details of footrot occurrence by sheep are presented in Appendix 3.

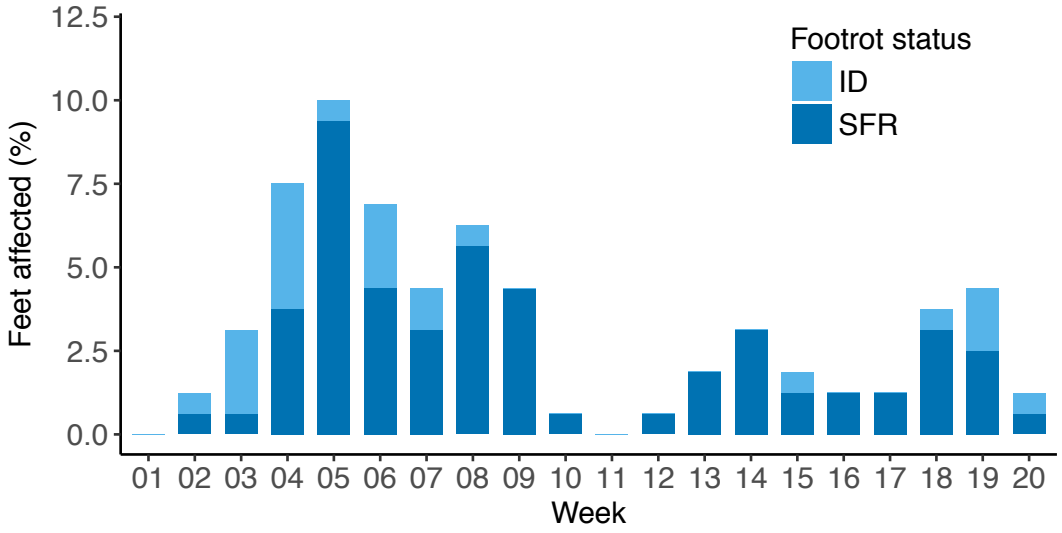


**Figure 3.7 Footrot prevalence during Study B**
The figure shows percentage of feet affected with ID and SFR by week.

### 3.4.2 Climate data from Study B

Mean temperature during the study ranged from 0.7°C to 26.8°C and increased during the study period. The average weekly rainfall was 9.1mm, however, from week 7 to week 11 (Figure 3.8) only 14.5mm of rain fell in total. This dry period preceded the period of lowest footrot prevalence (Figure 3.7, weeks 10-12). Soil temperature and moisture data are shown in Appendix 4.

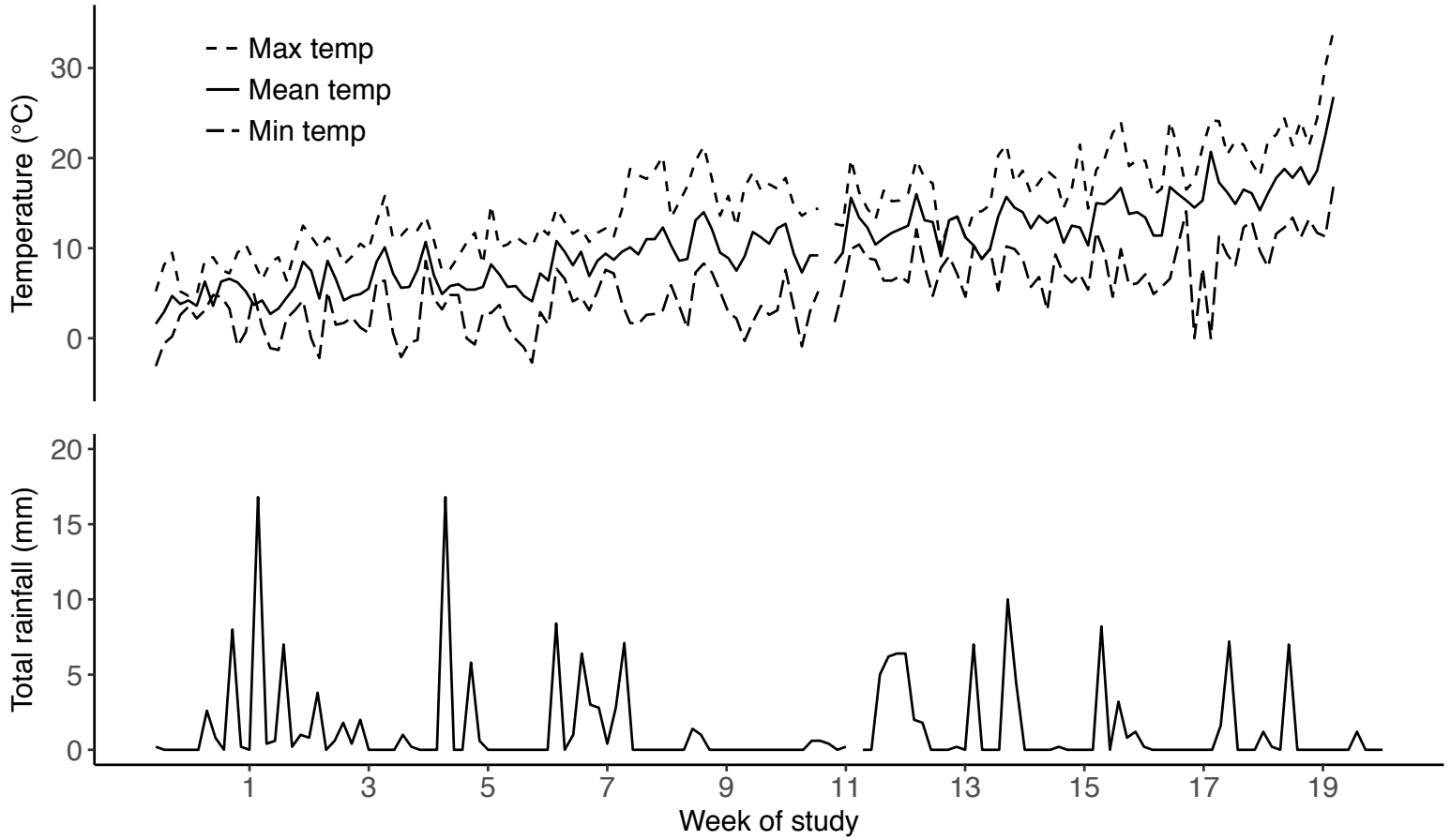


**Figure 3.8 Climate data for Study B**

Data from ten days prior to commencement of the study to the end of the study (week 20) are shown. Upper panel: max (maximum), mean and min (minimum) daily temperature in °C. Lower panel: daily rainfall in mm.

### 3.4.3 Detection and quantification of *Fusobacterium necrophorum* for Study B

DNA was extracted from 1136 foot swabs, 284 mouth swabs and 283 faecal samples from 30 sheep from the study (Section 3.2.6 and Appendix 3). Two DNA extraction batches (30 foot swabs) were excluded due to suspected contamination during the extraction process, giving a total of 1106 foot swabs. One faecal sample was mislabelled and therefore not analysed. DNA was extracted from all 640 environmental samples.

#### 3.4.3.1 Comparison of detection of *Fusobacterium necrophorum* between sample types

Overall, 5% of samples were positive for *F. necrophorum*. The distribution of positive samples across sample types was different than expected by chance ($p < 0.01$): as with Study A there were more positive foot and mouth samples, and fewer positive environmental samples than expected (Table 3.12).

**Table 3.12 Detection and load of *F. necrophorum* by sample type for Study B**

| Sample type | Frequency of detection | | *rpoB* copies in positive samples | |
|---|---|---|---|---|
| | No. | % | Minimum | Maximum |
| Foot swabs | 85/1106 | 7.7 | $1.03 \times 10^2$ swab$^{-1}$ | $8.50 \times 10^7$ swab$^{-1}$ |
| Mouth swabs | 21/284 | 7.4 | $1.82 \times 10^2$ swab$^{-1}$ | $1.67 \times 10^6$ swab$^{-1}$ |
| Faeces | 11/283 | 3.9 | $2.18 \times 10^5$ g$^{-1}$ [a] | $1.89 \times 10^7$ g$^{-1}$ |
| Soil | 4/462 | 0.9 | $6.52 \times 10^2$ g$^{-1}$ | $4.31 \times 10^3$ g$^{-1}$ |
| Grass | 0/178 | 0 | NA | NA |

[a] There was one rectal swab positive for *F. necrophorum* which had a load of $2.12 \times 10^3$ *rpoB* copies swab$^{-1}$

#### 3.4.3.2 Detection of *Fusobacterium necrophorum* over time

*F. necrophorum* was detected at all time points except weeks 18 and 20. Foot swabs were the only samples where *F. necrophorum* was detected after week 10, with the exception of one positive mouth swab in week 17. The highest detection rate on foot swabs occurred in week 1 (27.6% of swabs positive; Figure 3.9).

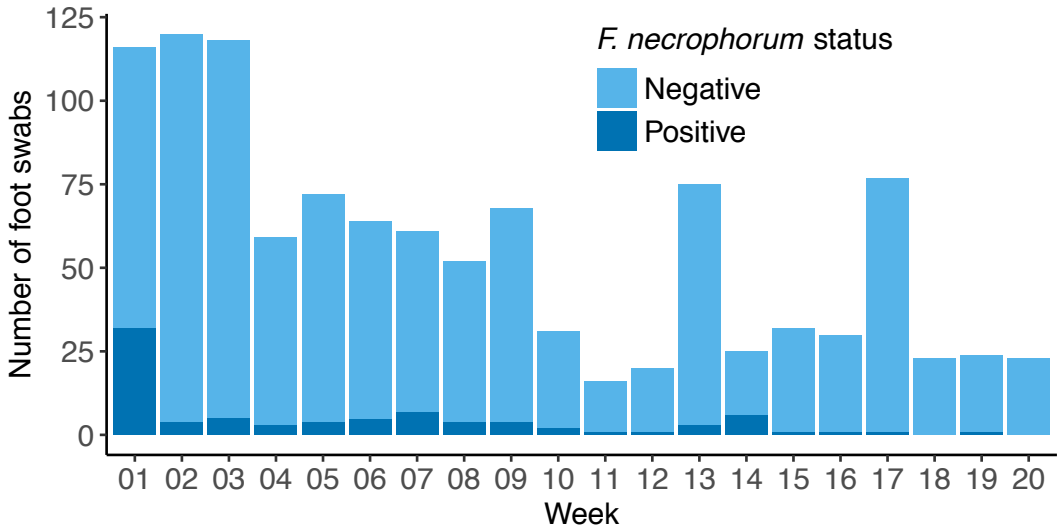


**Figure 3.9 Detection of *F. necrophorum* on foot swabs over time for Study B**

### 3.4.3.3 Detection and load of *Fusobacterium necrophorum* on foot swabs

Detection rates of *F. necrophorum* on foot swabs were highest in feet and sheep with SFR (Table 3.13). Foot swabs with higher loads of *F. necrophorum* were more likely to be from feet with ID or SFR than foot swabs with lower loads (OR 2.12, 95% CI 1.36-3.61; Figure 3.10).

**Table 3.13 Detection of *F. necrophorum* on foot swabs, mouth swabs and faecal samples by footrot status for Study B**

| | Healthy | | ID | | SFR | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| Foot swabs | | | | | | |
| *Sheep level* | 50/822 | 6.1 | 5/69 | 7.2 | 30/215 | 14.0 |
| *Foot level* | 62/1024 | 6.0 | 2/17 | 10.5 | 21/63 | 33.3 |
| Mouth swabs | 15/211 | 7.1 | 3/18 | 16.7 | 3/55 | 5.5 |
| Faecal samples | 9/210 | 4.3 | 1/18 | 5.6 | 1/55 | 1.8 |

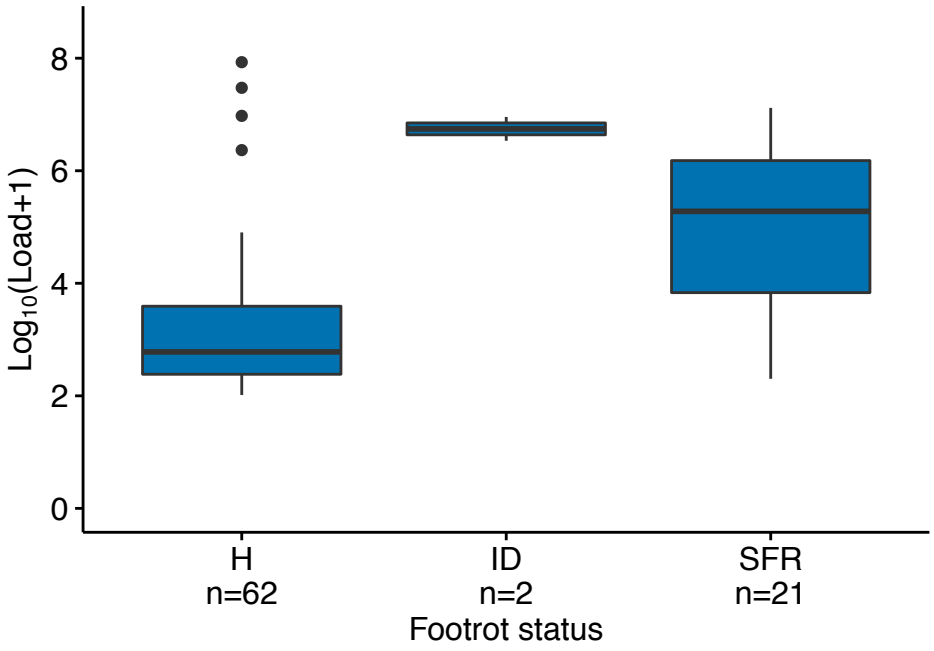


**Figure 3.10 Load of *F. necrophorum* on foot swabs by footrot status for Study B**

#### 3.4.3.4 Persistence of *Fusobacterium necrophorum* on feet

*F. necrophorum* was detected on the same foot for between 1 and 12 consecutive weeks, and was more likely to persist on feet that had footrot than those that were healthy ($p < 0.01$; Figure 3.11). Detection on individual feet over time is shown in Appendix 5.

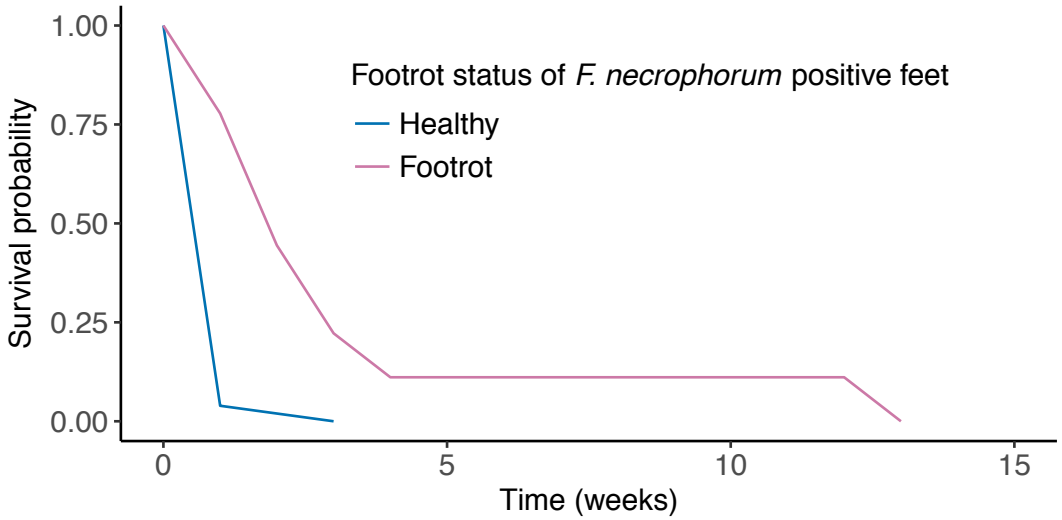


**Figure 3.11 Survival probability of *F. necrophorum* on feet for Study B**
The probability of feet positive for *F. necrophorum* remaining positive over time is plotted for feet that had footrot whilst positive and those that were healthy whilst positive.

#### 3.4.3.5 Detection and load of *Fusobacterium necrophorum* on mouth swabs and in faecal samples

Load of *F. necrophorum* on mouth swabs and in faecal samples was not associated with footrot status. Eight sheep had mouth swabs positive for *F. necrophorum* (Figure 3.12). Fifteen of twenty-one (71.4%) positive mouth swabs came from three sheep that had multiple positive mouth swabs (sheep 03520, 03463 and 03539; Figure 3.12). Ten of the eleven positive faecal samples were from two of these sheep (sheep 03520 and 03463; Figure 3.12). *D. nodosus* was not detected in the mouth swabs or faecal samples that were positive for *F. necrophorum* (data not shown).

#### 3.4.3.6 Persistence of *Fusobacterium necrophorum* in mouths and faeces

The longest period of consecutive detection of *F. necrophorum* on mouth swabs was 6 weeks (sheep 03539; Figure 3.12), and in faecal samples was 4 weeks (sheep 03520). The longest period from first to last positive mouth swab was 10 weeks, and from first to last positive faecal sample was 7 weeks (both sheep 03463).

### 3.4.3.7 Detection of *Fusobacterium necrophorum* in environmental samples

*F. necrophorum* was detected in soil but not grass, and this detection occurred only in the early part of the study: three of four positive soil samples were from the baseline samples taken 10 days before the sheep were moved onto the study pasture, and the fourth was from week 2. All positive soil samples were from the same high traffic area, a ring feeder; two were from surface soil (0-1cm) and two from deep soil (4-5cm).

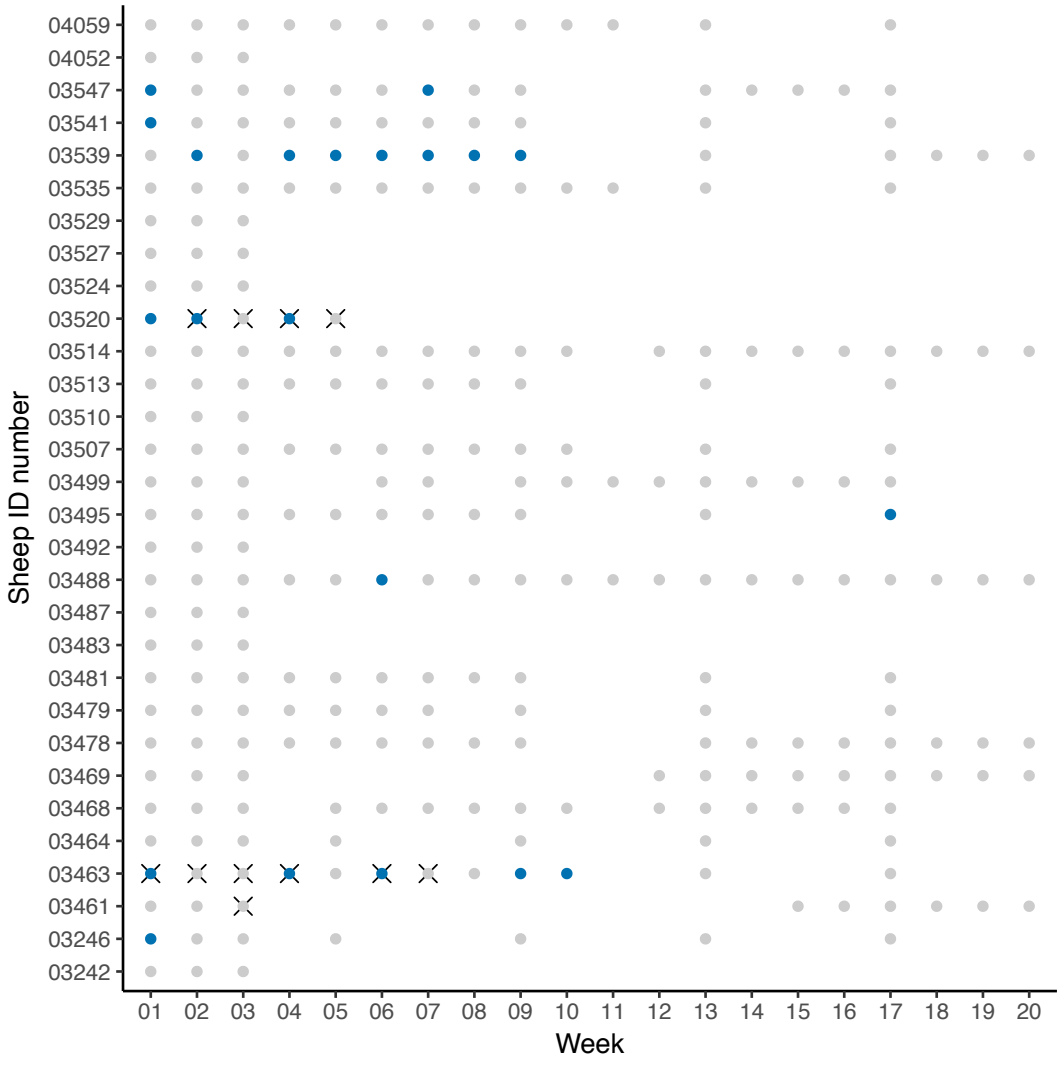


**Figure 3.12 Detection of *F. necrophorum* in mouth swabs and faecal samples by sheep for Study B**

Grey circles show samples analysed, blue circles show positive mouth swabs and black crosses show positive faecal samples.

### 3.4.4 Binomial mixed effects models of presence of *Fusobacterium necrophorum* on foot swabs

The results for the univariable analysis are shown in Table 3.14.

Explanatory variables with a p value < 0.1 were tested in a multivariable model using stepwise forward selection. Four explanatory variables were retained in the multivariable model (Table 3.15). The odds of a foot being positive for *F. necrophorum* increased as the load of *D. nodosus* and *F. necrophorum* on that foot one week previously increased (OR 1.65, 95% CI 1.33 - 2.09 and OR 1.48, 95% CI 1.17 - 1.86 respectively). A foot was more likely to be positive for *F. necrophorum* as minimum temperature during the previous week increased and maximum temperature during the previous week decreased (OR 1.40, 95% CI 1.04 - 1.97 and OR 0.79, 95% CI 0.65 - 0.97 respectively).

**Table 3.14 Univariable binomial mixed effects regression model of presence of *F. necrophorum* on foot swabs as determined by qPCR for Study B**

| Value one-week previously | No. | % | Odds ratio | Lower 95% CI | Upper 95% CI | P value < 0.1 |
|---|---|---|---|---|---|---|
| *Foot level* | | | | | | |
| $Log_{10}$ (Fn[a] load + 1) | 864 | 100 | 1.59 | 1.30 | 1.94 | * |
| $Log_{10}$ (Dn[a] load + 1) | 864 | 100 | 1.88 | 1.51 | 2.41 | * |
| Foot with footrot | 81 | 9.4 | 3.78 | 1.47 | 9.44 | * |
| ID score 0&1 | 842 | 97.5 | Ref | | | |
| ID score 2&3[b] | 22 | 2.5 | 4.87 | 1.10 | 20.4 | * |
| SFR score 0 | 801 | 92.7 | Ref | | | |
| SFR score 1 | 52 | 6.0 | 2.9 | 0.88 | 9.00 | * |
| SFR score 2&3[b] | 11 | 1.3 | 8.4 | 1.13 | 73.3 | * |
| *Sheep level* | | | | | | |
| Mouth swab Fn positive | 72 | 8.3 | 1.08 | 0.19 | 5.20 | |
| Faeces Fn positive | 36 | 4.2 | 2.56 | 0.22 | 42.1 | |
| Sheep with footrot | 276 | 31.9 | 1.62 | 0.76 | 3.43 | |
| Sheep positive for Fn on at least one foot | 224 | 25.9 | 5.31 | 2.34 | 11.7 | * |
| Sheep positive for Dn on at least one foot | 324 | 38 | 3.56 | 1.45 | 8.69 | * |
| *Week level* | | | | | | |
| Mean temp (°C) | 864 | 100 | 0.84 | 0.63 | 1.14 | |
| Min temp (°C) | 864 | 100 | 1.42 | 1.08 | 1.92 | * |
| Max temp (°C) | 864 | 100 | 0.79 | 0.72 | 0.93 | * |
| Total rainfall (mm) | 864 | 100 | 1.03 | 0.99 | 1.07 | * |
| Soil temp (°C) | 864 | 100 | 0.89 | 0.77 | 1.02 | * |
| Soil moisture (%) | 864 | 100 | 1.02 | 1.00 | 1.04 | * |
| Feet with Footrot[c] (%) | 864 | 100 | 1.07 | 0.87 | 1.31 | |
| Foot swabs positive[c] (%) | 864 | 100 | 0.98 | 0.91 | 1.04 | |
| Mouth swabs positive[c] (%) | 864 | 100 | 0.99 | 0.94 | 1.06 | |
| Faeces samples positive[c] (%) | 864 | 100 | 1.03 | 0.85 | 1.25 | |

Variables with p < 0.1 included in subsequent multivariable analysis are indicated by *. No. and % refer to the number and percentage of values in the dataset. CI = confidence interval. Ref = baseline category for comparison.

[a] Fn refers to *F. necrophorum* and Dn refers to *D. nodosus.* [b] Lesion scores 2 & 3 were grouped together due to low numbers of feet in each category. [c] Within flock

**Table 3.15 Multivariable binomial mixed effects regression model of presence of *F. necrophorum* on foot swabs as determined by qPCR for Study B**

| Value one-week previously | Odds ratio | Lower 95% CI | Upper 95% CI |
|---|---|---|---|
| *Fixed effects* | | | |
| $Log_{10}$ (Dn[a] load + 1) | **1.65** | 1.33 | 2.09 |
| $Log_{10}$ (Fn[a] load + 1) | **1.48** | 1.17 | 1.86 |
| Maximum temp (°C) | **0.79** | 0.65 | 0.97 |
| Minimum temp (°C) | **1.40** | 1.04 | 1.97 |
| | | | |
| Week[b] | 0.53 | 0.04 | 5.99 |
| $Week^2$ | 0.90 | 0.16 | 5.13 |
| $Week^3$ | 2.67 | 0.47 | 16.1 |
| $Week^4$ | 0.53 | 0.20 | 1.57 |
| *Random part* | | | |
| Variance (foot level) | 1.05 | | |
| Variance (sheep level) | 0.36 | | |

CI = confidence interval. Where odds ratios are in bold, they are statistically significant at 0.05 when CIs do not include unity.
[a] Fn refers to *F. necrophorum* and Dn refers to *D. nodosus.* [b] Mean centered term for week

#### 3.4.4.1 Correlations and associations between explanatory variables

Associations for variables retained in the multivariable model are shown in Tables 3.16 and 3.17. Associations between all explanatory variables are shown in Appendix 6. Among explanatory variables in the final model, the load of *F. necrophorum* and *D. nodosus* were significantly positively correlated, and the load of *D. nodosus* was significantly positively correlated with minimum temperature. Minimum and maximum temperature were strongly positively correlated.

All four variables in the final model were positively correlated with the foot having footrot, and increasing SFR score. Minimum and maximum temperature were both positively correlated with the percentage of feet with footrot in the flock, and negatively correlated with the percentage of mouths and faeces positive for *F. necrophorum* in the flock. Maximum temperature was negatively correlated with the percentage of feet positive for *F. necrophorum* in the flock.

There was a strong positive correlation between both minimum and maximum temperature and soil temperature. Maximum temperature was negatively correlated with soil moisture. Load of *F. necrophorum* was positively correlated with weekly rainfall and soil moisture, and negatively correlated with mean temperature. Load of *D. nodosus* was positively correlated with weekly rainfall.

**Table 3.16 Associations with continuous and ordinal variables included in the final multivariable model for Study B**

| | Spearman correlation coefficient | | | |
|---|---|---|---|---|
| *Variables included in the final model* | 1 | 2 | 3 | 4 |
| 1. $Log_{10}$ (Dn[a] load + 1) | | | | |
| 2. $Log_{10}$ (Fn[a] load + 1) | **0.35** | | | |
| 3. Maximum temp (°C) | 0.03 | -0.05 | | |
| 4. Minimum temp (°C) | **0.08** | -0.02 | **0.71** | |
| | | | | |
| *Other continuous/ordinal variables* | | | | |
| Foot ID score | **-0.07** | -0.06 | 0.03 | -0.02 |
| Foot SFR score | **0.14** | **0.24** | **0.15** | **0.09** |
| Mean temperature (°C) | -0.01 | **-0.11** | **0.95** | **0.81** |
| Total rainfall (mm) | **0.08** | **0.12** | -0.38 | -0.01 |
| Soil temperature (°C) | 0.03 | -0.06 | **0.91** | **0.71** |
| Soil moisture (%) | 0.06 | **0.11** | **-0.68** | -0.31 |
| Feet with footrot[b] (%) | **-0.08** | **-0.12** | **0.28** | **0.08** |
| Foot swabs positive[b] (%) | **0.1** | **0.23** | **-0.17** | -0.03 |
| Mouth swabs positive[b] (%) | 0.02 | **0.1** | **-0.53** | **-0.21** |
| Faeces samples positive[b] (%) | **-0.12** | -0.06 | **-0.66** | **-0.6** |

Table shows coefficients for Spearman correlation. Coefficients in bold represent significant associations.

[a] Fn refers to *F. necrophorum* and Dn refers to *D. nodosus.* [b] Within flock

**Table 3.17 Associations with binary variables included in the final multivariable model for Study B**

| | Direction of association[a] | | | |
|---|---|---|---|---|
| *Binary variables* | 1 | 2 | 3 | 4 |
| Foot with footrot | + | + | + | + |
| Sheep with footrot | | + | + | + |
| Sheep positive for Fn on at least one foot | + | + | - | |
| Sheep positive for Dn on at least one foot | + | + | | + |
| Mouth swab Fn positive | | | - | |
| Faecal sample Fn positive | - | | - | - |

Only significant associations are shown.

[a] + indicates a positive association and - indicates a negative association. 1-4 are as shown in Table 3.16 (1 = $Log_{10}$ (Dn load + 1), 2 = $Log_{10}$ (Fn load + 1), 3 = Maximum temp, 4 = Minimum temp).

### 3.4.5 Linear mixed effects models of load of *Fusobacterium necrophorum* on foot swabs

Results for the univariable model are shown in Table 3.18.

Two variables were retained in the final linear model (Table 3.19): feet with ID score 2 & 3 were more likely to have higher loads of *F. necrophorum* the following week than feet with ID0 or ID1 (β = 2.35, 95% CI 0.92 – 3.78), and loads of *F. necrophorum* on feet increased with increasing maximum temperature during the previous week (β = 0.26, 95% CI 0.07 – 0.44).

**Table 3.18 Univariable linear mixed effects regression model of $log_{10}$(load + 1) of *F. necrophorum* on foot swabs as determined by qPCR for Study B**

| Value one-week previously | No. | % | β | Lower 95% CI | Upper 95% CI | P value < 0.1 |
|---|---|---|---|---|---|---|
| *Foot level* | | | | | | |
| $Log_{10}$ (Fn[a] load + 1) | 53 | 100 | 0.34 | 0.17 | 0.51 | * |
| $Log_{10}$ (Dn[a] load + 1) | 53 | 100 | 0.13 | -0.09 | 0.35 | |
| Foot with footrot | 18 | 34.0 | 1.66 | 0.63 | 2.69 | * |
| ID score 0&1 | 48 | 90.6 | Ref | | | |
| ID score 2&3[b] | 5 | 9.4 | 2.30 | 0.77 | 3.82 | * |
| SFR score 0 | 38 | 71.7 | Ref | | | |
| SFR score 1 | 9 | 17.0 | 0.15 | -1.16 | 1.47 | |
| SFR score 2&3[b] | 6 | 11.3 | 1.5 | -0.54 | 3.54 | |
| *Sheep level* | | | | | | |
| Mouth swab Fn positive | 3 | 5.7 | -0.69 | -2.86 | 1.48 | |
| Faeces Fn positive | 2 | 3.8 | -0.14 | -3.60 | 3.32 | |
| Sheep with footrot | 26 | 49.1 | 0.61 | -0.42 | 1.63 | |
| Sheep positive for Fn on at least one foot | 36 | 67.9 | 0.77 | -0.29 | 1.83 | |
| Sheep positive for Dn on at least one foot | 37 | 69.8 | -0.07 | -1.38 | 1.25 | |
| *Week level* | | | | | | |
| Mean temp (°C) | 53 | 100 | 0.12 | -0.29 | 0.52 | |
| Min temp (°C) | 53 | 100 | -0.34 | -0.68 | 0.01 | * |
| Max temp (°C) | 53 | 100 | 0.25 | 0.05 | 0.45 | * |
| Total rainfall (mm) | 53 | 100 | -0.04 | -0.08 | 0.01 | * |
| Soil temp (°C) | 53 | 100 | 0.18 | 0.01 | 0.34 | * |
| Soil moisture (%) | 53 | 100 | -0.02 | -0.05 | 0.01 | |
| Feet with Footrot[c] (%) | 53 | 100 | -0.01 | -0.30 | 0.27 | |
| Foot swabs positive[c] (%) | 53 | 100 | 0.04 | -0.05 | 0.13 | |
| Mouth swabs positive[c] (%) | 53 | 100 | -0.02 | -0.10 | 0.06 | |
| Faeces samples positive[c] (%) | 53 | 100 | 0.03 | -0.23 | 0.28 | |

Variables with p < 0.1 included in subsequent multivariable analysis are indicated by *.
No. and % refer to the number and percentage of values in the dataset.
β = coefficient. CI = confidence interval. Ref = baseline category for comparison.
[a] Fn refers to *F. necrophorum* and Dn refers to *D. nodosus.* [b] Lesion scores 2 & 3 were grouped together due to low numbers of feet in each category. [c] Within flock

**Table 3.19 Multivariable linear mixed effects regression model of $log_{10}$(load + 1) of *F. necrophorum* on foot swabs as determined by qPCR for Study B**

| Value one-week previously | β | Lower 95% CI | Upper 95% CI |
|---|---|---|---|
| *Fixed effects* | | | |
| ID score 0&1 | Ref | | |
| ID score 2&3[a] | **2.35** | 0.92 | 3.78 |
| Maximum temp (°C) | **0.26** | 0.07 | 0.44 |
| | | | |
| Week[b] | -0.42 | -2.37 | 1.54 |
| Week$^2$ | 0.53 | -1.24 | 2.30 |
| Week$^3$ | -0.12 | -1.99 | 1.75 |
| Week$^4$ | -0.16 | -1.29 | 0.97 |
| *Random part* | | | |
| Variance (foot level) | 0.38 | | |
| Variance (sheep level) | $5.13 \times 10^{-9}$ | | |

β = coefficient. CI = confidence interval. Ref = baseline category for comparison. Where odds ratios are in bold, they are statistically significant at 0.05 when CIs do not include unity.

[a] Lesion scores 2 & 3 were grouped together due to low numbers of feet in each category.

[b] Mean centered term for week.

#### 3.4.5.1 Correlations and associations between explanatory variables

Associations for maximum temperature were presented in Section 3.4.4.1 above. ID score was positively associated with variables relating to footrot occurrence at the foot, sheep and flock level. It was negatively correlated with load of *F. necrophorum* and *D. nodosus* on feet although the correlation coefficients were small (r = -0.07 and r = -0.06 respectively).

## 3.5 Discussion

This is the first longitudinal study of presence and load of *F. necrophorum* in sheep and their environment. Study A was a small group of sheep in a situation where footrot was active, and Study B was a group of 40 healthy sheep that were separated onto a clean pasture. Footrot incidence and prevalence in Study B remained low throughout the study. This may have been due to the dry weather during the study, with a total rainfall of 190mm from Feb – Jun 2015 compared with a range of 227 – 332mm in the previous three years. Footrot transmission increases with wetter conditions (Graham and Egerton, 1968). In addition, separating a group of forty healthy sheep effectively controlled footrot by reducing force of infection (Green *et al.*, 2007; Russell *et al.*, 2013b). Study B therefore provides useful information regarding *F. necrophorum* persistence during low footrot prevalence.

### 3.5.1 *Fusobacterium necrophorum* presence and persistence in the environment of sheep

*F. necrophorum* was detected at low frequency in soil in both Study A and Study B (8% and 0.9% respectively), and was only detected on one occasion on grass. These detection rates do not fit those that would be expected for a pathogen with significant environmental reservoirs, for example in a longitudinal study of dairy cattle an environmental mastitis pathogen, *Streptococcus uberis*, was detected in over 60% of environmental samples (Zadoks *et al.*, 2005).

Across the two studies detection of *F. necrophorum* in soil occurred primarily in high traffic areas, suggesting contamination of the environment by sheep. Detection levels in feet and the environment decreased concurrently in Study A, and in Study B detection occurred in soil in week 2 following high levels of detection on feet in week 1. Outbreaks of necrobacillosis in other ungulates are reported in connection with animals gathering at feed or watering stations during periods of high rainfall (Monrad *et al.*, 1983; Edwards *et al.*, 2001; Handeland *et al.*, 2010), suggesting that the environment supports transient

presence of *F. necrophorum* when conditions are favourable for transmission. These studies also highlight that under more extensive grazing systems and without heavy rainfall, necrobacillosis in ungulates is sporadic. This provides further evidence that soil is not a normal site for *F. necrophorum* persistence, and that transmission via the environment is generally low. Further study of high traffic areas during periods of high rainfall would be needed to determine if the environment has a significant role in transmission of *F. necrophorum* between sheep in wet conditions.

Overall, the evidence from these two studies is that *F. necrophorum* is shed onto pasture by infected sheep, and that its survival on pasture is transient. This is a complete paradigm change from the previous assumption that *F. necrophorum* is ubiquitous in the environment of sheep, and that the environment represents a significant reservoir for footrot.

### 3.5.2 *Fusobacterium necrophorum* presence and persistence on the feet of sheep

Feet were the only site where *F. necrophorum* was consistently detected over the entire study period for Study B, suggesting that feet were the primary site for persistence of *F. necrophorum* within this flock. *F. necrophorum* was more likely to persist on feet with footrot than healthy feet, and the majority of healthy feet were only positive for 1 week. This suggests that although *F. necrophorum* can be detected on healthy feet, they are only transiently positive and therefore unlikely to represent a significant site of persistence.

Increased loads of *F. necrophorum* were found in feet with footrot in both studies and in Study B feet with ID were more likely to have higher loads of *F. necrophorum* the next week. This supports previous evidence from Witcomb *et al.* (2014), that *F. necrophorum* load increases once footrot has occurred and not before. This is an important distinction, as it implies that footrot facilitates *F. necrophorum* growth, rather than *F. necrophorum* facilitating footrot as previously suggested (Roberts & Egerton, 1969).

Individuals shedding levels of an organism above a certain threshold are referred to as "super shedders" (Omisakin *et al.*, 2003; Cobbold *et al.*, 2007). Feet (and therefore sheep) with footrot have high loads of *F. necrophorum* for extended periods of time, and could therefore be considered as super shedders. It has generally been assumed that super shedders are important for pathogen transmission, however, recent work for both *E. coli* O157:H7 and *Mycobacterium avium* subsp. *paratuberculosis* in cattle faeces suggests that for these bacteria, super shedders have minimal effects on transmission (2-3 fold increase in risk of transmission) despite shedding quantities of bacteria several orders of magnitude higher than low shedding animals (Spencer *et al.*, 2015; Slater *et al.*, 2016). Further work would be necessary to understand the effect of sheep with footrot on *F. necrophorum* transmission within a flock.

Overall the data from foot swabs suggest that *F. necrophorum* is more likely to persist and multiply on feet with footrot. This is a key finding as it provides the first evidence for the role of footrot in maintenance of *F. necrophorum* populations within a flock.

### 3.5.3 *Fusobacterium necrophorum* presence and persistence in sheep faeces

Detection of *F. necrophorum* in faeces was infrequent in both Study A and Study B. In Study B, where faecal samples were collected directly from sheep, 3/30 sheep shed high loads of *F. necrophorum* in faeces for periods of between 1 and 4 consecutive weeks. This is the first direct evidence that *F. necrophorum* can be shed in sheep faeces, but suggests that shedding is not widespread amongst sheep.

In 2 of the 3 sheep that shed *F. necrophorum* in faeces, the length of the shedding period could not be fully determined because shedding was still occurring at the last sample analysed. Further study would be needed to understand if shedding is a transient property, as suggested by Spencer *et al.* (2015), that could occur in any individual, or if it is specific to certain individuals based on *F. necrophorum* being a stable member of the GI microbiota in these sheep and not others.

### 3.5.4 The role of climate in *Fusobacterium necrophorum* persistence

There was an association between environmental temperature and both the likelihood of a foot being positive for *F. necrophorum* and load of *F. necrophorum* on positive feet. The load of *F. necrophorum* on feet increased as maximum temperature increased, and this may reflect an effect of temperature on the growth rate of *F. necrophorum*. Graham and Egerton (1968) showed that environmental temperatures below 10°C reduce the temperature and blood supply of the feet of sheep, and proposed that this would affect bacterial growth on feet.

Feet were more likely to be positive for *F. necrophorum* at higher minimum temperatures but at lower maximum temperatures. This implies that *F. necrophorum* survival and transmission increased when temperatures were less extreme. Evidence from Australia showed that footrot transmission did not occur below 50°F (10°C), or during hot dry periods (Graham & Egerton, 1968), however evidence from the UK suggests that footrot transmission can occur year round despite temperatures frequently falling below 10°C (Ridler *et al.*, 2009). Moisture is reported to be the most important environmental factor for footrot transmission (Graham & Egerton, 1968), and the conditions during Study B were unseasonably dry as discussed earlier. This may have made bacterial survival more sensitive to temperature than would be the case under wetter conditions, and further study would be needed to determine if temperature was as influential during periods of high rainfall. Finally, it should be noted that there were only 19 values present in the regression models for each temperature variable, and this may have reduced the reliability of the parameter estimates for these variables.

### 3.5.5 *Fusobacterium necrophorum* presence and persistence in the mouths of sheep

Detection rates of *F. necrophorum* in mouths differed by approximately ten-fold between the two studies, and this was due to a difference in the number of sheep that had repeated detections at this site (10/10 in Study A and 3/30 in Study B). The difference in detection rates of *F. necrophorum* from mouth swabs between the two studies could relate to the difference in footrot prevalence (maximum 20% feet affected in Study A

compared to 10% in Study B), however, *F. necrophorum* detection and load were not associated with footrot status in either study. In Study A there was some indication that sheep with footrot were more likely to have a positive mouth swab two weeks later, but a larger number of samples would be needed to confirm this. Alternatively, if detection levels were unrelated to footrot, it may be that *F. necrophorum* is present in the mouth as part of the oral microbial community in some sheep. Fusobacteria form part of the oral cavity microbiome in humans, with variation in this community observed between individuals (Yang *et al.*, 2012). In both studies presented here there was evidence that *F. necrophorum* could persist in the mouth, which would support this theory. A study across a larger number of farms would be needed to determine if the differences in proportion of sheep with persistent detection observed between the two studies presented here was due to chance, or whether this is representative of a real pattern across sheep farms.

### 3.5.6 Improvements for future studies

The low footrot prevalence and transmission in Study B provided the opportunity to study the sites at which *F. necrophorum* persisted under these conditions. However, the low frequency of footrot lesions made it difficult to determine associations between load and disease prevalence, severity and chronicity.

Separate binomial and linear mixed effects regression models were used to analyse qPCR data from foot swabs, however a zero-inflated model could be used to combine the analysis of presence/absence and load data. The models used provided information regarding variables that were associated with presence and load of *F. necrophorum*, but a transition model could be used to investigate variables associated with changes of state from negative to positive and vice versa. Autoregression could be used to allow for varying degrees of correlation between repeated samples from the same foot over time.

Due to constraints on time and expenditure, a subset of samples from the 40 sheep in Study B was selected for analysis. Analysis of the remaining samples would give a more complete picture of persistence and transmission of *F. necrophorum* within the flock. If

the complete set of samples were analysed, mathematical modelling could be used to understand transmission dynamics as demonstrated for other pathogens (Spencer *et al.*, 2015; Slater *et al.*, 2016).

### 3.5.7 Conclusions from Chapter 3

Longitudinal, quantitative data for *F. necrophorum* demonstrated that contrary to prior assumption, the environment was not a significant reservoir of *F. necrophorum* in these studies. Instead, *F. necrophorum* persisted in sheep, primarily on feet but also in the mouths and faeces of certain individuals. Feet were the most consistent site for *F. necrophorum* persistence in a flock, and footrot facilitated persistence at this site. *F. necrophorum* was able to persist in mouths for at least 8 weeks, and could be persistently shed in faeces for at least 4 weeks. Further evidence is needed to understand the significance of these sites for transmission of *F. necrophorum* to feet.

# Chapter 4 Development and validation of a multiple locus variable number tandem repeat analysis (MLVA) scheme for *Fusobacterium necrophorum*

This chapter consists of a paper that has been accepted for publication in the journal Veterinary Microbiology. Farm A in this paper is Study A as described in Chapter 3.

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## 4.1 Abstract

*Fusobacterium necrophorum* is associated with various diseases in humans and animals. It is believed to persist in reservoirs when not associated with disease but for most diseases the reservoir is unknown. Strain typing of *F. necrophorum* would facilitate linking a specific reservoir with a specific disease. The aim of this study was to develop multiple locus variable number tandem repeat analysis (MLVA) as a strain typing technique for *F. necrophorum*, and to test the use of this scheme to analyse both isolates and mixed communities. Seventy-three tandem repeat regions were identified in the *F. necrophorum* genome; three of these loci were suitable and developed as a MLVA scheme. The MLVA scheme was sensitive, specific, and discriminatory for both isolates and communities of *F. necrophorum*. The MLVA scheme strain typed 46/52 *F. necrophorum* isolates including isolates of both subspecies and from different countries, host species and sample sites within host. There were 12 unique MLVA strain types that clustered by subspecies. The MLVA scheme characterised the *F. necrophorum* community in DNA from 32/49 foot- and 28/33 mouth swabs from sheep. There were 17 community

types in total. In 31/32 foot swabs, single strains of *F. necrophorum* were detected while in the 28 mouth swabs there were up to a maximum of 8 strains of *F. necrophorum* detected. The results demonstrate the potential of this method to elucidate reservoirs of *F. necrophorum.*

**Key words:** *Fusobacterium necrophorum*, community, strain typing, MLVA

## 4.2 Introduction

*Fusobacterium necrophorum* is associated with a variety of diseases, termed necrobacilloses, in humans and animals. In humans, *F. necrophorum* causes Lemierre's disease (Lemierre, 1936; Riordan, 2007; Kuppalli *et al.*, 2012) and is associated with pharyngitis (Aliyu *et al.*, 2004; Ludlam *et al.*, 2009), periodontal disease (Enwonwu *et al.*, 1999; Gomes *et al.*, 2004; Jacinto *et al.*, 2008) and appendicitis (Rogers *et al.*, 2016). In animals, *F. necrophorum* causes hepatic abscesses that occur in intensively reared beef cattle (Lechtenberg *et al.*, 1988; Narayanan *et al.*, 1997; Nagaraja & Chengappa, 1998) and it is associated with footrot in sheep (Egerton *et al.*, 1969; Witcomb *et al.*, 2014), foot infections in other ungulates (Clark *et al.*, 1985; Edwards *et al.*, 2001; Handeland *et al.*, 2010), endometritis in cattle (Ruder *et al.*, 1981), calf diphtheria (Panciera *et al.*, 1989), respiratory disease in deer (Brooks *et al.*, 2014) and periodontal disease in wallabies (Antiabong *et al.*, 2013b).

*F. necrophorum* is considered to be an opportunistic pathogen (Langworth, 1977; Tan *et al.*, 1996), consequently reservoirs of the bacterium are assumed to be present in healthy individuals and/or their environment. However, there has been little research to confirm that reservoirs in healthy individuals exist. In cattle, strain typing was used to identify the bovine rumen as the reservoir of *F. necrophorum* that causes hepatic abscesses (Narayanan *et al.*, 1997). In humans, *F. necrophorum* was thought to be part of the throat microflora of healthy individuals (Lemierre, 1936; Bartlett & Gorbach, 1976), however, it has only been detected in people aged 18-39, although Lemierre's disease and other *F. necrophorum* infections can occur at any age (Aliyu *et al.*, 2004; Jensen *et al.*, 2007; Ludlam *et al.*, 2009). In sheep, *F. necrophorum* has been isolated from the gingiva (McCourtie *et al.*, 1990; Bennett *et al.*, 2009) and detected on both healthy and footrot-diseased feet (Witcomb *et al.*, 2014), but the significance of these sites as reservoirs is unknown. Whilst *F. necrophorum* has been widely assumed to be ubiquitous in sheep faeces and soil (Marsh & Tunnicliff, 1934; Roberts & Egerton, 1969; Langworth, 1977; Winter, 2004a) this is unsubstantiated.

There are two subspecies of *F. necrophorum*: *necrophorum* and *funduliforme*. These are distinguished by a PCR assay to detect a haemagglutinin-related gene that is present in subsp. *necrophorum* but not *funduliforme* (Narongwanichgarn *et al.*, 2003). To confidently identify reservoirs associated with specific diseases, strain typing of *F. necrophorum* over time is needed, as exemplified by Narayanan *et al.* (1997). Multiple locus variable number tandem repeat analysis (MLVA) is an objective, repeatable, PCR-based strain typing method that has been used in a variety of epidemiological studies of bacterial pathogens (Wada *et al.*, 2007; Vranckx *et al.*, 2011; Eyre *et al.*, 2013; Halkilahti *et al.*, 2013; Russell *et al.*, 2013a; Mezal *et al.*, 2014). MLVA was originally developed to analyse individual isolates but it can also be used to analyse samples that may contain a mixed community of strains within a species (Vranckx *et al.*, 2011). In these cases, MLVA is used to produce a molecular "fingerprint" of the strains present and so identify similarities and differences between communities.

The aim of the current study was to develop an MLVA typing scheme for *F. necrophorum*, and to demonstrate its potential to analyse isolates and community DNA. A selection of *F. necrophorum* isolates from a variety of host species and countries, together with DNA extracted from swab samples from the feet and mouths of sheep, were used to develop and validate the scheme.

## 4.3 Materials and methods

### 4.3.1 Identification of tandem repeat regions for MLVA analysis

Seventy-three tandem repeat regions (Appendix 7) were identified from the whole genome shotgun sequence of *F. necrophorum* ATCC 51357 (GenBank Accession number AJSY00000000.1) using the Tandem Repeats Finder software v.4.08 (Benson, 1999). Nine regions were excluded due to insufficient flanking sequence to facilitate PCR primer design for amplification of the target region. There were 34 regions identified using blastn (Altschul *et al.*, 1990), where flanking sequences were present in all of the three published *F. necrophorum* genomes available (accessed March 2014). PCR primers targeting the 3' and 5' flanking regions of these 34 repeat regions were designed using BatchPrimer3 v1.0 (You *et al.*, 2008). Eight *F. necrophorum* subsp. *necrophorum* isolates (Appendix 7) were tested first for amplification of the target region and then for polymorphism at the tandem repeat region. Three loci (Fn13, Fn42 and Fn69; Appendix 7) showed good amplification and sufficient polymorphism for use in MLVA typing. PCR primers used to amplify the three selected MLVA targets and their tandem repeat sizes are given in Table 4.1.

**Table 4.1 Primers identified to develop MLVA PCR for *Fusobacterium necrophorum***

| Target | Repeat size (bp) | Primers | Sequence (5' to 3')[a] |
|---|---|---|---|
| *F. necrophorum* ATCC 51357 contig 2 | 17 | Fn13(F) | 6FAM™-AATTCAAAATGATTTCTCCCTACCT |
| | | Fn13(R) | TGAGAAAGAAGATAAATGGAAAACG |
| *F. necrophorum* ATCC 51357 contig 11 | 11 | Fn42(F) | PET®-TTCCCAAAATAGCAGAAAAACATAC |
| | | Fn42(R) | ACCGAAAATTCAATATCAAAATCAA |
| *F. necrophorum* ATCC 51357 contig 4 | 12 | Fn69(F) | NED™-TTGATTATCCATTTTCCTTTTTGAC |
| | | Fn69(R) | CAATCCTACCTCGATTATTTCTTCA |

[a] Sequence of forward primer includes fluorescent marker attached to 5' end. Fluorescently labelled forward primers were sourced from Applied Biosystems, Warrington, UK; reverse primers from Sigma-Aldrich Ltd., Gillingham, UK.

### 4.3.2 MLVA PCR reactions and cycling conditions

PCR reactions were carried out in a final volume of 25µl and contained 12.5µl Bioline MyTaq™ Red Master Mix (2×; Bioline Reagents Ltd., London, UK), 1µl molecular biology grade bovine serum albumin (BSA; 100µg $ml^{-1}$; Sigma-Aldrich Ltd., Gillingham, UK), 1µl each of forward and reverse primers (10µM; Table 4.1), and 1µl template DNA. In reactions using mixed DNA, 1µl betaine (5M; Sigma-Aldrich Ltd., Gillingham, UK) was also included to improve sensitivity.

Cycling conditions were 95°C for 5 min, followed by 32 cycles of 94°C for 30 sec, 55°C (Fn13 and Fn69) or 62°C (Fn42) for 30 sec, 72°C for 30 sec, followed by final extension at 72°C for 10 min. All PCR reactions were carried out on an Eppendorf Mastercycler ep gradient machine (Eppendorf, Hamburg, Germany) with DNA extracted from *F. necrophorum* subsp. *necrophorum* DSM 21784 as the positive control and nuclease free $H_2O$ as the reagent blank. PCR products were visualized after ethidium bromide-stained agarose gel electrophoresis and imaged using a Gene Flash imager (Syngene Bio Imaging, Cambridge, UK).

### 4.3.3 Validation of the MLVA typing scheme

PCR primer specificity was tested using DNA from a selection of non-target organisms (*Fusobacterium gonidiaformans* [DSM 19810], *Fusobacterium nucleatum* subsp. *polymorphum* [DSM 20482], *Dichelobacter nodosus* [VCS1703A], *Mycobacterium bovis* [BCG], *Escherichia coli, Mannheimia* sp., *Pseudomonas* sp., *Staphylococcus epidermidis, Staphylococcus intermedius,* and *Streptococcus uberis;* all from University of Warwick).

The sensitivity of amplification for each loci was tested using a ten-fold dilution series from $10^6$ to $10^1$ genome copies $\mu l^{-1}$ of *F. necrophorum* DSM 21784 DNA added to DNA extracted from *F. necrophorum* negative sheep foot swabs (Witcomb *et al.*, 2014). The number of genome copies in the stock DNA was calculated based on the genome size for *F. necrophorum* subsp. *funduliforme* (2,088,497 bp; Calcutt *et al.*, 2014). A blank

containing DNA extracted from *F. necrophorum* negative foot swabs was run alongside the dilution series.

The stability of the MLVA scheme was tested by comparing the MLVA strain type of two *F. necrophorum* isolates before and after ten passages of culture on Fusobacterium Agar, a selective medium based on that used by Brazier *et al.* (1991) (Wilkins-Chalgren Anaerobe Agar with Gram-negative Anaerobe Selective Supplement (both Oxoid Ltd., Altrincham, UK), 5% defibrinated sheep blood and josamycin (3μg $ml^{-1}$)).

### 4.3.4 Determining PCR amplicon size using fragment analysis

The size, in base pairs, of PCR products was determined using fragment analysis: samples were submitted to DNA Sequencing and Services™ (College of Life Sciences, University of Dundee, UK) and results analysed with Peak Scanner 2 Software (Applied Biosystems, Warrington, UK). Sanger sequencing of the PCR products from each of the three assays for *F. necrophorum* DSM 21784 were used as a reference for the number of repeats to be calculated from the size in base pairs for each sample. A variation in expected size of PCR amplicon of ±2bp was tolerated.

### 4.3.5 MLVA typing of *Fusobacterium necrophorum* isolates

A total of 52 isolates, 43 *F. necrophorum* subsp. *necrophorum* and 9 *F. necrophorum* subsp. *funduliforme,* were used in this study. The country and sites of origin of the isolates are listed in Table 4.2.

**Table 4.2 Country, animal host, site of sample and subspecies of 52 *F. necrophorum* isolates tested by MLVA**

| Country | Animal | Site | Subspecies | Number of isolates |
|---|---|---|---|---|
| UK | Sheep | Foot | *necrophorum* | 9 |
| | | Mouth | *funduliforme* | 1 |
| | Cattle | Liver abscess | *necrophorum* | 8 |
| | | | *funduliforme* | 1 |
| USA | Cattle | Liver abscess | *necrophorum* | 9 |
| | | | *funduliforme* | 4 |
| | | Footrot | *necrophorum* | 6 |
| | | Rumen | *necrophorum* | 1 |
| | | | *funduliforme* | 3 |
| | Elk | Footrot | *necrophorum* | 4 |
| France | Sheep | Foot | *necrophorum* | 1 |
| Spain | Sheep | Foot | *necrophorum* | 5 |

Isolates were cultured on Fusobacterium agar (as above) and then sub-cultured on Wilkins-Chalgren Anaerobe Agar (Oxoid Ltd., Altrincham, UK) with 5% defibrinated sheep blood. All incubations were carried out under anaerobic conditions (Don Whitley MACS-MG-1000 anaerobic workstation; 80% $N_2$, 10% $CO_2$ and 10% $H_2$, Don Whitley Scientific Ltd., Shipley, UK) at 30°C for 2-5 days. DNA was extracted from cultures using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Ltd., Manchester, UK) according to the manufacturer's instructions with a lysis time of 1 hour. A *F. necrophorum* specific PCR targeting the gyrase B gene (Jensen *et al.*, 2007) was used to confirm that isolates were *F. necrophorum,* and amplification of the haemagglutinin-related protein gene used to confirm isolates as subspecies *necrophorum* rather than subspecies *funduliforme* (Narongwanichgarn *et al.*, 2003).

The strain type of *F. necrophorum* isolates was determined by the number of repeats at each of the three loci (Fn13, Fn42 and Fn69) after PCR and fragment analysis. Each strain type was assigned a unique number. The Hunter-Gaston Discriminatory Index (HGDI) for the strain typing scheme was calculated (Hunter & Gaston, 1988) with 95% confidence intervals (Grundmann *et al.*, 2001). Minimum-spanning trees for the isolate strain typing

data were created in PHYLOViZ-2.0 (Francisco *et al.*, 2012) using the global optimal eBURST (goeBURST) distance algorithm with Euclidean distance (Francisco *et al.*, 2009). The population was grouped on single locus variants (SLV).

### 4.3.6 MLVA typing of *Fusobacterium necrophorum* communities from swab samples

Initially a model community was made by combining equal concentrations of DNA from four isolates of *F. necrophorum* that between them contained three variants at both Fn13 and Fn42, and two variants at Fn69. This was then tested to investigate whether, in a mixed community, all the variants at each locus were detected using the MLVA typing scheme.

DNA was extracted from 82 swabs (33 mouth and 49 foot swabs) taken from sheep on six farms (A – F) in England (Table 4.3) as described by Purdy (2005). Those confirmed positive for *F. necrophorum* using the *rpoB* qPCR as per Witcomb *et al.* (2014) were used for MLVA community analysis. On Farm A, samples were collected as part of a longitudinal study: 10 sheep were sampled every 2 weeks for 8 weeks. On Farms B – F, 15 sheep were sampled per farm on one occasion.

**Table 4.3 Location, date and swab site (mouth or feet) from six sheep farms**

| Farm | Location by county in England | Month(s) and year sampled | Frequency of detection of *F. necrophorum* | | | |
|---|---|---|---|---|---|---|
| | | | Feet | | Mouths | |
| | | | No. | % | No. | % |
| A | Warwickshire | 05-07/2014 | 76/152 | 50 | 30/38 | 79 |
| B | Norfolk | 11/2015 | 2/13 | 15 | 7/15 | 47 |
| C | Cheshire | 12/2015 | 2/13 | 15 | 1/15 | 7 |
| D | West Midlands | 01/2016 | 3/14 | 21 | 7/15 | 47 |
| E | Staffordshire | 01/2016 | 3/16 | 19 | 1/15 | 7 |
| F | Warwickshire | 01/2016 | 2/14 | 14 | 1/15 | 7 |

For the swab samples, the number of MLVA variants within a locus was determined by fragment analysis. The minimum number of strains in a community was calculated as equal to the greatest number of MLVA variants at one locus. The maximum number of strains detected in a community was calculated by multiplying the number of variants at

each locus together (e.g. if a sample contains 1, 2 and 3 variants for the three loci, the minimum number of strains is 3 and the maximum is 6 (1 x 2 x 3)). Each unique pattern of MLVA variants within these samples was assigned a unique “community type” number. The HGDI and associated confidence interval were calculated based on the frequency of detection of each community type.

## 4.4. Results

### 4.4.1 Validation of PCR amplification of the loci

The PCR assays for the three MLVA loci (Fn13, Fn42 and Fn69) were specific, with no PCR product produced from any of the non-target organisms tested. The detection limit was $10^4$ genome copies $\mu l^{-1}$ of extracted DNA for the Fn13 assay, and $10^3$ genome copies $\mu l^{-1}$ of extracted DNA for the Fn42 and Fn69 assays. The MLVA scheme was stable; the MLVA type of the two isolates matched their original MLVA type after ten culture passages.

### 4.4.2 Population diversity of *Fusobacterium necrophorum* isolates

The three MLVA loci were characterised in 46/52 (88%) *F. necrophorum* isolates. The 6 isolates that were not fully characterised were excluded from further analysis. In the fully characterised isolates there were three variants at locus Fn13, five at Fn42 and four at Fn69 (Table 4.4) giving 12 unique MLVA strain types (Appendix 8), 6 of which were detected only once. The HGDI for the strain typing scheme was 0.85 (95% CI 0.80 – 0.90), so that two distinct strains would be characterised as different on 85% of occasions.

**Table 4.4 Size in base pairs of PCR amplicon, number of repeats and frequency of detection of locus variants of 46 *F. necrophorum* isolates**

| Variant name[a] | Size in bp | Number of repeats | Frequency of detection |
|---|---|---|---|
| 13.1 | 463 | 1 | 12 |
| 13.1a | 508 | 1 | 8 |
| 13.2 | 479 | 2 | 26 |
| 42.2 | 475 | 2 | 5 |
| 42.3 | 487 | 3 | 1 |
| 42.4 | 499 | 4 | 1 |
| 42.5 | 511 | 5 | 22 |
| 42.6 | 523 | 6 | 17 |
| 69.2 | 470 | 2 | 16 |
| 69.3 | 482 | 3 | 24 |
| 69.4 | 494 | 4 | 4 |
| 69.4a | ~1300[b] | 4 | 2 |

[a] 13.1a and 69.4a indicates the presence of additional unrelated DNA sequence within the PCR amplicon which produced an anomalous sized PCR product.
[b] The 69.4a variant was too large to be identified on fragment analysis using the standard size marker and was therefore identified on gel electrophoresis

Strain types varied within subspecies, country, host species and sample site. The goeBURST analysis detected 2 groups (Figure 4.1): 11/12 strains were in a major group with strain types 3 (n=9) and 7 (n=2) the predicted ancestral strains. Strain type 5 (n=5) was in an unconnected group by itself. Both sub-species were present in both groups and were clearly clustered within the major group (Figure 4.1A); only 1/11 strain types in the major group contained both subspecies. There was no clear clustering of strains by host species (Figure 4.1B), country of origin (Figure 4.1C), or tissue site (Figure 4.1D). The variation in strains indicates that analysis of a greater number of isolates could provide evidence of clustering if it exists.

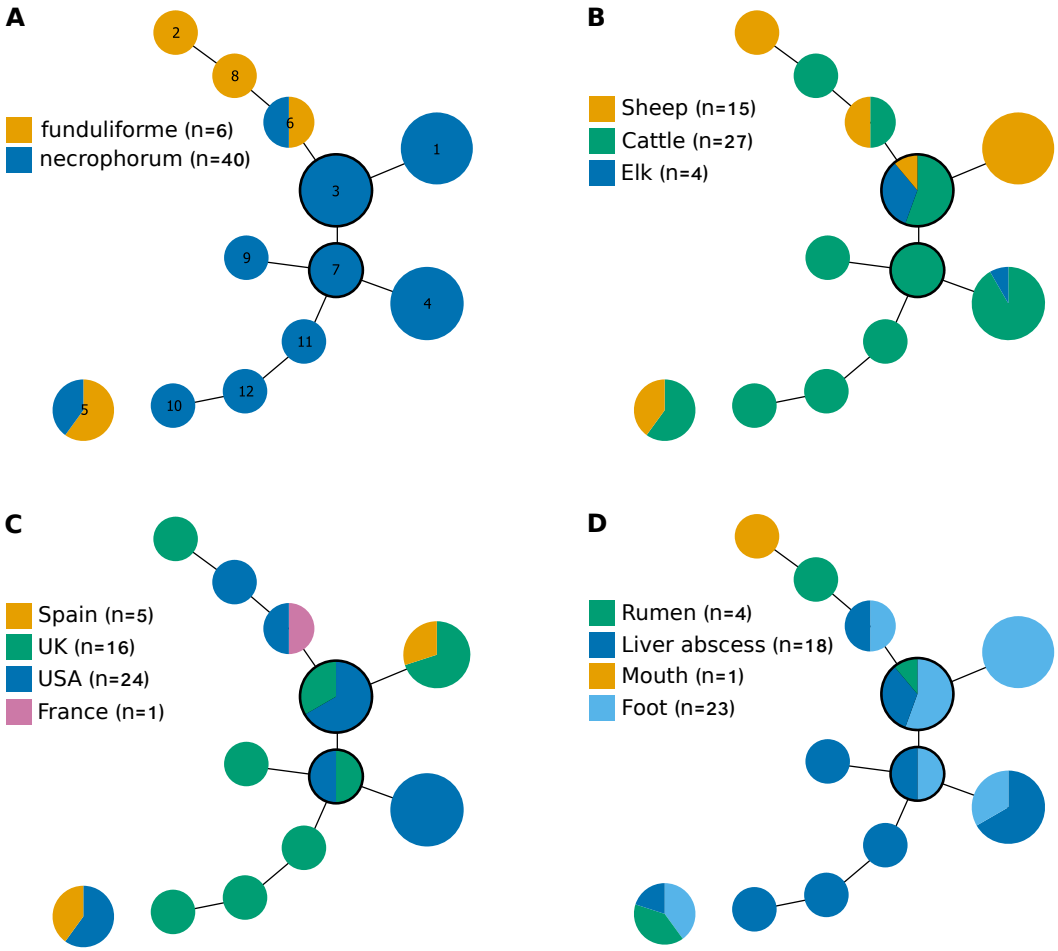


**Figure 4.1 Analysis of *Fusobacterium necrophorum* MLVA strain type clustering using goeBurst**

Single locus variants are connected by solid lines. Numbers indicate MLVA strain type, and size of circle represents number of isolates of each MLVA type. Types 3 and 7 are the suggested founder strain types, indicated by the black border. The shading indicates isolates of (A) different subspecies, (B) different host species, (C) different countries of origin, and (D) different sites of origin. Individual isolates are not always in the same position within a circle between the 4 trees, the coloured sections are placed with the most frequently represented first from the 12 o'clock position.

### 4.4.3 Community diversity of *Fusobacterium necrophorum* in DNA from swab samples

All expected locus variants were detected in the model community (data not shown) indicating that the MLVA scheme was able to detect strains in mixed communities of *F. necrophorum*. All three MLVA loci were amplified from 28/33 (85%) mouth and 32/49 (65%) foot swab samples (Table 4.5).

**Table 4.5 Proportion of MLVA community analysis amplified by farm and site of swab**

| Farm | No. swabs with community type determined/no. swabs analysed | | | |
|---|---|---|---|---|
| | Foot swabs | | Mouth swabs | |
| | No. | % | No. | % |
| A | 25/37 | 68 | 15/16 | 94 |
| B | 0/2 | 0 | 6/7 | 86 |
| C | 0/2 | 0 | 1/1 | 100 |
| D | 3/3 | 100 | 5/7 | 71 |
| E | 2/3 | 67 | 1/1 | 100 |
| F | 2/2 | 100 | 0/1 | 0 |

There were 17 unique community types (Appendix 8), 10 of which contained more than 1 strain, these ranged from a minimum of 2 to a maximum of 8 strains (Table 4.6).

**Table 4.6 Minimum and potential maximum numbers of strains present in community types with multiple strains**

| Community type | Farm | Potential number of strains | |
|---|---|---|---|
| | | Minimum[a] | Maximum[b] |
| 2 | A | 2 | 2 |
| 3 | A | 2 | 4 |
| 4 | A | 2 | 4 |
| 6 | A | 2 | 2 |
| 9 | D | 3 | 6 |
| 11 | B | 2 | 8 |
| 12 | B | 2 | 2 |
| 13 | B | 2 | 2 |
| 14 | B | 2 | 2 |
| 16 | B | 2 | 2 |

Community types 1, 5, 7, 8, 10, 15 and 17 contained only one strain of *F. necrophorum.*
[a] Minimum potential strains = number of detected variants at the most diverse locus.
[b] Maximum potential strains = number of variants at each locus multiplied together.

The *F. necrophorum* communities in mouth swabs were more complex than the communities in foot swabs. There were 16 community types in mouth swabs; the overall HGDI was 0.94 (95% CI 0.90 - 0.98). There were only 4 community types in foot swabs; 31/32 (97%) foot swabs had a single strain type (one of strain types 1, 3 and 6 from Figure 4.1) consequently the HGDI was not calculated for foot swabs.

The locus variants from the 10 sheep from Farm A are presented in Figure 4.2. The same strain was detected over time and 24/25 foot swabs were a single strain (strain type 1 in the isolate analysis (Figure 4.1)) rather than a community of *F. necrophorum*. The three locus variants in this strain (13.2, 42.5 and 69.2) were also detected in mouths in sheep 5, 7 and 8, indicating that this strain was potentially present in mouths. There were, however, many more strain types in mouths than feet. In mouths, some locus variants and community types were stable over time for example, the same community type was detected at all four time points in sheep 3 and 7 and in sheep 5 and 10 the community types differed by one additional locus variant present in 50% of the samples.

Community data from Farms B-F is presented in Figure 4.3. As with Farm A (Figure 4.2), complex communities (up to 8 strains in Sheep 1 Farm B (Figure 4.3)) were present in mouth swabs whereas only single strains of *F. necrophorum* were detected in foot swabs.

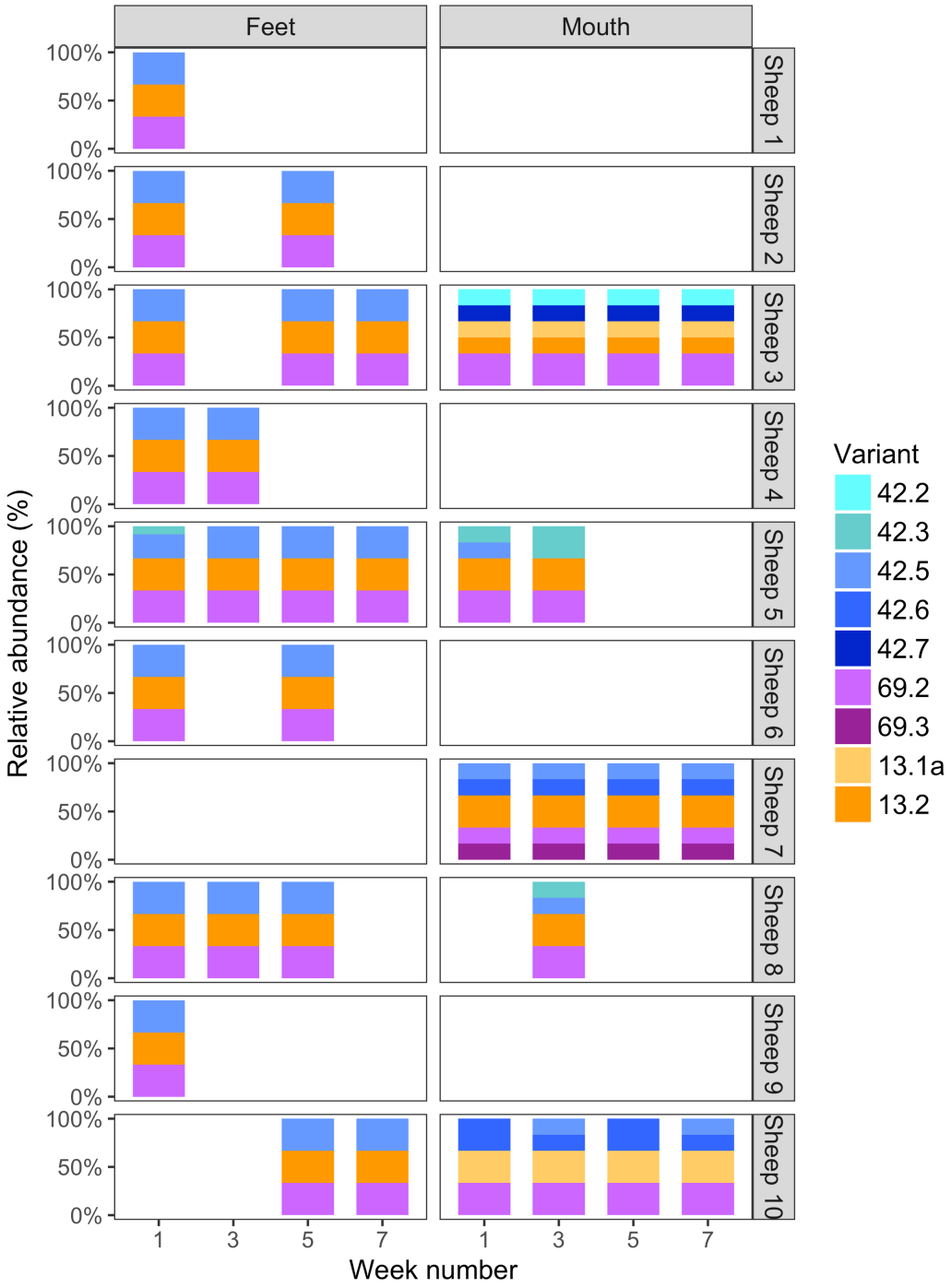


**Figure 4.2 Relative abundance of locus variants in swab samples from Farm A**
The ten sheep from Farm A are listed on the right of the figure. Results from all positive foot swabs from a sheep (sometimes >1 positive per sheep) are represented in the left-hand panels, and mouths in the right. Note, in all but one of the sheep (sheep 5, week 1) all positive samples contained the same community type, which was represented by a single strain type (strain type 1 in Figure 4.1).

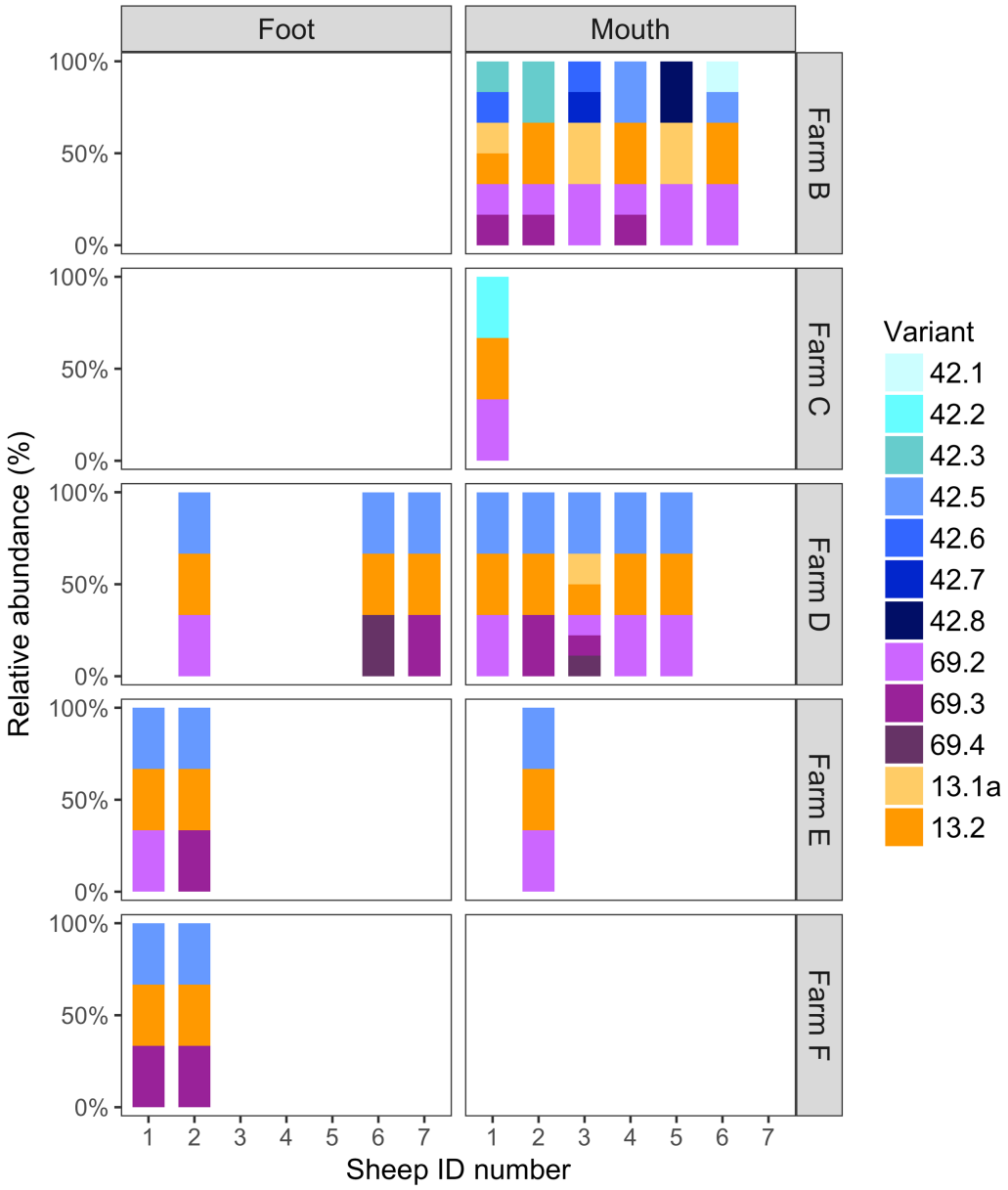


**Figure 4.3 Relative abundance of locus variants in swab samples from Farms B-F**
The five farms are indicated by the letters B-F on the right of each panel. Feet are represented in the left-hand panels, and mouths in the right. The sheep identification number is indicated on the *x* axis.

## 4.5 Discussion

The MLVA typing scheme developed for *F. necrophorum* was specific and sensitive with the potential to strain type isolates and community DNA. Discriminatory ability, stability, epidemiological concordance, typeability and reproducibility are also used to evaluate typing schemes (van Belkum *et al.*, 2007). A HGDI discriminatory value of ≥0.95 is recommended for typing schemes (van Belkum *et al.*, 2007). The discriminatory ability of this 3-loci scheme was 0.85 (95% CI 0.80 – 0.90) for isolates and 0.94 (95% CI 0.90 - 0.98) for communities of *F. necrophorum*. Whilst ideally we would have liked to make the scheme more discriminatory there were no more loci appropriate for the scheme. The results from isolates and communities do suggest that the scheme is sufficiently discriminatory for these samples. The identification of the same strain type for two isolates after multiple passages through culture demonstrated the stability of the scheme. Finally, there was good epidemiological concordance for the scheme, for example, single strains were detected on the feet of sheep over time on Farm A, whilst more complex and varied communities were detected in mouth samples over the same time.

A wide range of *F. necrophorum* isolates from three ruminant hosts and four countries was used to develop the scheme. The MLVA scheme was sufficiently discriminatory to differentiate isolates from the same country, host, site and subspecies. There was no clustering of *F. necrophorum* strain types by country, host or site from the isolates analysed. This might be due to the relatively small number of isolates analysed or because provenance of the samples meant that there were no clusters in the dataset. Clusters might be detectable in a dataset specifically selected to investigate the host disease and its complimentary reservoir, e.g. as reported by Narayanan *et al.* (1997) for liver abscesses and the rumen reservoir in the same host animal.

This is the first study of communities of *F. necrophorum* in sheep and provides pilot data for further study. The communities in the mouth were more complex than on the feet. There were locus variants in mouths that were never detected on feet from sheep on the

same farm, suggesting site-specificity for some strains. In contrast, the strain of *F. necrophorum* detected on feet was potentially (i.e. its 3 loci were present) in the mouths of some sheep on Farm A, suggesting that the mouth could be a reservoir or a spill-over site from feet. With the exception of one sample, only single strains of *F. necrophorum* were detected on feet. The consistency over time and the discriminatory power of the MLVA scheme suggest that this is likely to be a true reflection of the samples analysed. The generalisability of this pattern of very limited diversity on feet is unknown, however, Zhou *et al.* (2009) also reported the presence of single strains of *F. necrophorum* in 14 DNA samples extracted from foot swabs from sheep.

It is likely that there were loci variants that were not detected in the community DNA samples in the current study because of the limit of detection of the PCR. This may have affected locus Fn13 more because detection of this locus is less sensitive than Fn42 and 69. This limits the use of the scheme for community samples to those with *F. necrophorum* loads of more than ~$10^4$ copies per µl of extracted DNA. Improvements in the sensitivity of detection at this locus would enable the analysis of a wider range of samples.

## 4.6 Conclusions

A sensitive, specific, stable and discriminatory MLVA typing scheme was developed and validated for both isolates and community DNA samples of *F. necrophorum*. Using samples from sheep, the scheme is epidemiologically plausible and has potential to improve understanding of reservoirs of *F. necrophorum* and their association with necrobacilloses in both non-human and human animals.

## 4.7 Acknowledgements

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# Chapter 5 Use of a *Fusobacterium necrophorum* MLVA community typing scheme to analyse samples from Study B

## 5.1 Introduction

The results presented in Chapter 4 demonstrated the use of the *F. necrophorum* MLVA scheme to understand variation within and between communities of *F. necrophorum* in mouths and on feet of sheep. The analysis of samples from Study A in Chapter 4 provided valuable information regarding persistence of *F. necrophorum* at these sites, and potential transfer between sites. In Chapter 5 I present MLVA analysis of foot swabs, mouth swabs and faecal samples from Study B (Section 3.4.3).

## 5.2 Methods

DNA samples extracted from 79 foot swabs, 19 mouth swabs and 11 faecal samples that were positive for *F. necrophorum* from Study B (Section 3.4.3) were analysed using the MLVA typing scheme described in Section 4.3.

## 5.3 Results

### 5.3.1 Community diversity of *Fusobacterium necrophorum*

A full or partial (one or two loci amplified) MLVA profile was obtained from 68/109 (62%) samples (Table 5.1). A full profile was obtained from 24/79 (30%) foot swabs, 4/19 (21%) mouth swabs and 2/11 (18%) faecal samples. Partial profiles were generated from 16 foot swabs, 13 mouth swabs and 9 faecal samples. From the 30 samples with a full MLVA profile, 6 community types were identified; 2 (types 18 and 19) had not been identified previously (Section 4.4.3). Community types could not be determined from samples with partial profiles.

From samples analysed, there were 2 variants detected at locus Fn13, and 4 variants at each of loci Fn42 and Fn69 (Table 5.1). At locus Fn13 and locus Fn42 one variant was found

on 94% (31/33) and 95% (54/57) of samples respectively, whereas at locus Fn69 the most frequently detected variant was found on 55% (31/56) of samples (Table 5.1). Only 1 faecal and 1 foot sample had > 1 strain. The details of the variants detected in each individual sample are shown in Appendix 9.

**Table 5.1 Frequency of detection of locus variants at sample sites in Study B**

| MLVA Variant | Foot swabs (n=40) | Mouth swabs (n=17) | Faecal samples (n=11) | Total |
|---|---|---|---|---|
| *Locus Fn13* | | | | |
| 13.1a | 1 (0)[a] | 1 | 0 | 2 |
| 13.2 | 23 (15) | 5 | 3 | 31 |
| *Locus Fn42* | | | | |
| 42.4 | 1 (0) | 0 | 0 | 1 |
| 42.5 | 32 (16) | 13 | 9 | 54 |
| 42.6 | 0 | 1 | 0 | 1 |
| 42.7 | 1 (0) | 0 | 0 | 1 |
| *Locus Fn69* | | | | |
| 69.1 | 0 | 1 | 0 | 1 |
| 69.2 | 0 | 9 | 5 | 14 |
| 69.3 | 24 (14) | 1 | 6 | 31 |
| 69.4 | 8 (0) | 2 | 0 | 10 |

[a] Number detected (number from feet with footrot)

### 5.3.2 Variation at locus Fn69 by sample site

The majority of variation occurred at locus Fn69 for all sites, therefore this locus was used to study variation between feet, sheep and over time.

#### 5.3.2.1 Variation at locus Fn69 on foot swabs

Variant Fn69.3 and Fn69.4 were detected on foot swabs (Table 5.1). Fn69.4 was detected on feet from weeks 1 – 3 but never for more than one week. Fn69.3 was detected on feet from week 4 and was detected on consecutive weeks on 6 feet (Figure 5.1). Different variants were detected on the same foot at different points in the study on three occasions, these were left rear of sheep 03468 week 1 and 13, left fore of sheep 03478 week 1 and 6, and right fore of sheep 03478 week 3 and 7 (Figure 5.1). The same variant was detected on multiple feet of the same sheep on 5 occasions; these were variant

Fn69.4 in sheep 03463 week 2, and variant Fn69.3 in sheep 03468 week 13, sheep 03478 week 7, and sheep 03535 weeks 8 and 9 (Figure 5.1).

#### 5.3.2.2 Variation at locus Fn69 on mouth swabs and in faecal samples

All 4 variants were detected from mouth swabs and variants Fn69.2 and Fn69.3 were detected from faeces (Table 5.1). Variants Fn69.1 and Fn69.4 were only detected for one week but variant Fn69.2 was detected on consecutive weeks from both faeces and mouth swabs, and variant Fn69.3 was detected on consecutive weeks from faeces (Figure 5.1). There was a change in variant over time within sheep on 3 occasions: sheep 03463 faeces week 4-6, sheep 03539 mouth week 8-9 and sheep 03647 mouth week 1 and 7 (Figure 5.1). Fn69.2 was detected in the mouth and faeces of the same sheep on one occasion; this was sheep 03463 in week 1. Fn69.4 was detected in the mouth and on a foot of the same sheep on one occasion; this was sheep 03547 in week 1. The same variant was not detected in the faeces and on a foot of a sheep at any time point (Figure 5.1).

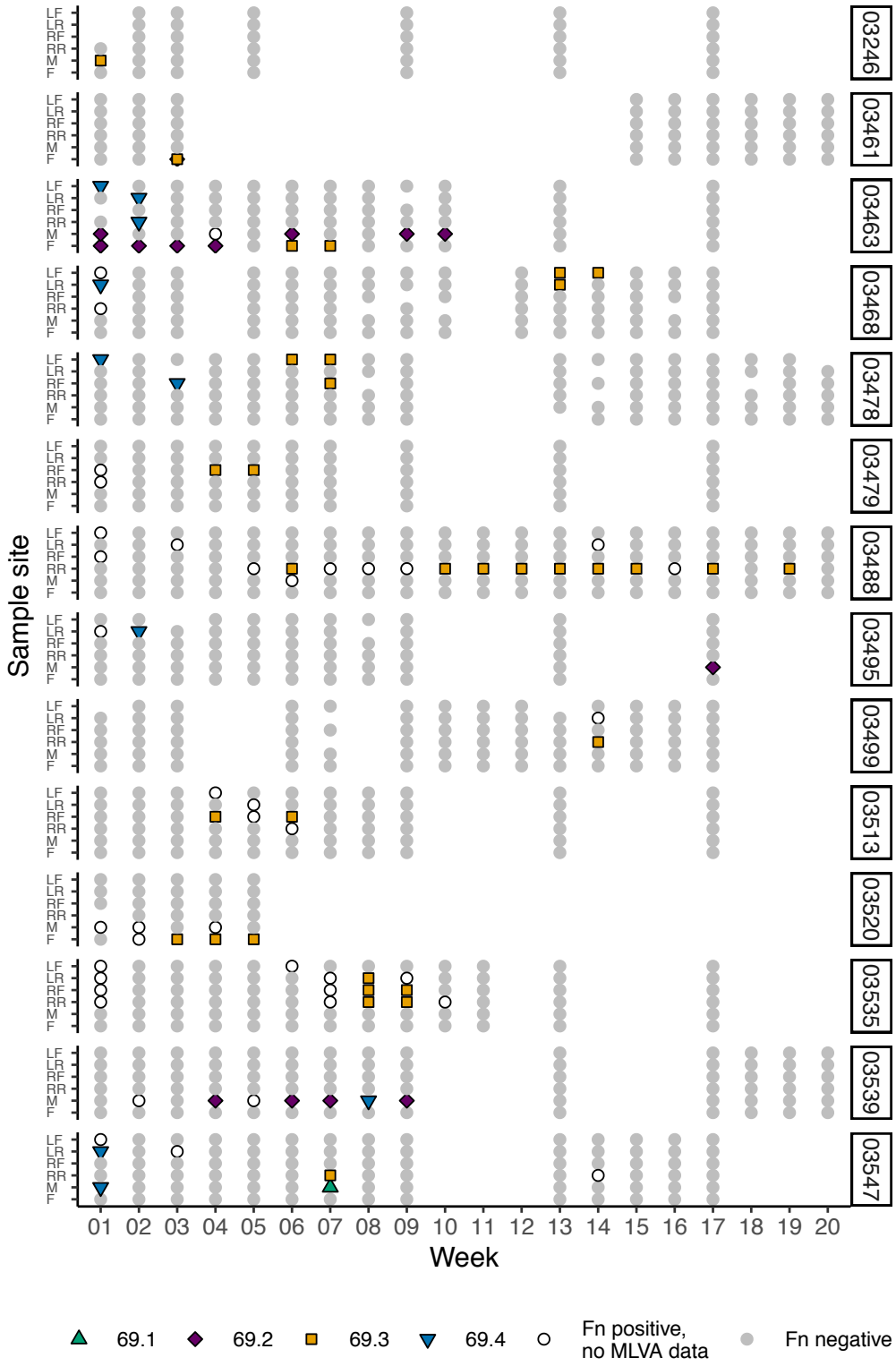



**Figure 5.1 Detection of locus Fn69 variants over time and by sheep for Study B**
Sheep ID number right hand panel, sample site on the *y* axis (LF = left fore, LR = left rear, RF = right fore, RR = right rear, M = mouth, F = faeces). Fn = *F. necrophorum*. The faeces sample from sheep 03461 in week 3 contained variants 69.2 and 69.3.

### 5.3.3 Comparison of *Fusobacterium necrophorum* strains detected on foot swabs between farms

Given the predominance of single strain communities on the feet of sheep (Sections 4.4.3 and 5.3.1), the MLVA strains detected in Study B were compared with those in Study A, and the five farms from a cross sectional study described in Section 4.4.3. There were four strains detected across the seven farms, three of which varied from each other only at locus Fn69 (Table 5.2).

**Table 5.2 Strains of *F. necrophorum* detected on foot swabs from Study A, Study B and five farms from a cross sectional study**

| Community type number[a] | Variant (number of repeats) by locus | | | Number of times detected | | |
|---|---|---|---|---|---|---|
| | Fn13 | Fn42 | Fn69 | Study A | Study B | CS[b] |
| 1 | 2 | 5 | 2 | 24 | 0 | 2 |
| 8 | 2 | 5 | 3 | 0 | 21 | 4 |
| 17 | 2 | 5 | 4 | 0 | 1 | 1 |
| 18 | 1 | 5 | 4 | 0 | 1 | 0 |

Only foot swabs with a complete MLVA profile are included.
[a] The method for determining community type is described in Section 4.3.6.
[b] Five farms were sampled as part of a cross sectional study described in Section 4.3.6.

## 5.4 Discussion

The single strains of *F. necrophorum* detected on foot swabs from the seven farms studied in Chapters 4 and 5 were closely related by MLVA profile. There were strains of *F. necrophorum* detected in mouths and faeces that were never detected on feet. These findings suggest that the strains detected on feet may share characteristics that make them well adapted to this site and that some of the strains in mouths and faeces may not survive on feet. Strain by site variation may occur because of differences in pathogenicity or the different environment on feet compared with mouths and faeces, for example, differences in temperature, moisture, and pH. Greater knowledge of the characteristics of different strains of *F. necrophorum* would improve our understanding of the role of populations of *F. necrophorum* as a reservoir for disease, not only in footrot but also in other necrobacilloses.

In Study B, Fn69.4 was detected on feet at the start of the study and disappeared after week 3; a second variant, Fn69.3, then became dominant on feet. One hypothesis for this is that the change of strain was associated with separation of the study group from the main flock onto clean pasture and that the first strain was carried on feet but did not persist. In mouths and faeces, the same strains persisted throughout the study. This pattern might indicate that flock management can perturb populations of *F. necrophorum* on feet but not mouths and faeces. This hypothesis is based on the acceptance that MLVA distinguishes strains and so different variants represent different strains of *F. necrophorum,* and that whilst MLVA was not successful on 100% of occasions, lack of success was random across strains.

Fn69.3 was detected on several occasions in faeces, on healthy feet and on feet with footrot, and therefore strains containing Fn69.3 may have been transmitted from faeces to feet, or between feet. There were two findings that suggest transmission between feet was more likely. Firstly, if transmission of *F. necrophorum* strains from faeces to feet occurred, detection of the Fn69.2 variant that was frequent in faeces would also be expected on feet, however Fn69.2 was never detected on feet. Therefore, either the strains containing the Fn69.2 variant were unable to survive on feet, or faeces were not a frequent source of *F. necrophorum* on feet. Secondly, it would be expected that if faeces were a frequent source of *F. necrophorum* on feet, a sheep shedding *F. necrophorum* in faeces would be more likely to have the same strain on her feet. Sheep 03463 is the only sheep where these data are available, and different Fn69 variants were detected on the feet and in faeces of this sheep (Figure 5.1), supporting the theory that transmission from faeces to feet was less common than transmission between feet.

Fn69.3 was only detected once from mouth swabs, which implies that mouths were not an important reservoir of *F. necrophorum* for feet. A number of mouth swabs (n=7) were *F. necrophorum* positive but with no MLVA data for locus Fn69, and it is therefore possible

that Fn69.3 was present but not detected in these mouth swabs. However, only two of these swabs were from the same sheep on consecutive weeks, and therefore even if these 7 swabs contained Fn69.3 it would still suggest that the mouth was a transient reservoir. In contrast, Fn69.2 was detected on consecutive occasions from mouth swabs but never on feet. The mouth may therefore be a site of persistence for strains of *F. necrophorum* not found on feet.

There was no evidence that communities containing several strains of *F. necrophorum* were present in mouths in Study B. This contrasts with Study A, where mouth swabs contained several strains of *F. necrophorum* that were stable over time and specific to individual sheep. It is unclear why *F. necrophorum* communities in the mouths of sheep were so markedly different between the two flocks. There is no evidence from existing literature on the acquisition and development of the microbial community in the oral cavity of sheep. In humans the oral cavity microbial community is mainly derived from the microbial communities of the mother, and is then modified by factors including diet and the external environment (Gomez & Nelson, 2017). If the same is true for sheep, it is possible that differences between farms in diet and bacteria present in the farm environment could influence the oral cavity community in sheep on that farm; this requires further investigation.

### 5.4.1 Improvements and future work

Incomplete MLVA profiles for samples in Study B made it difficult to determine patterns of persistence and transmission. Currently, the evidence indicates that the strains of *F. necrophorum* on feet are intermittently present in mouths and faeces. Improving the sensitivity of the MLVA would clarify whether or not the mouth and faeces are reservoirs for *F. necrophorum* strains also present on feet. In addition, determining the variant present on the *F. necrophorum* positive but MLVA negative foot swabs would clarify whether variant Fn69.4 faded out and Fn69.3 became the dominant variant at this time.

The findings from the current study are based on variation at one MLVA locus, Fn69. There is almost certainly more variation in *F. necrophorum* than detected from this single locus. The identification of more loci to add to the 3 in the current MLVA, or the use of genome sequencing would increase understanding of the strains of *F. necrophorum,* their habitat(s), and behaviour over time.

### 5.4.2 Conclusions from Chapter 5

Community typing of *F. necrophorum* positive DNA samples from Study B using MLVA revealed that *F. necrophorum* was persistently detected in the mouths and faeces of sheep and that the variants of *F. necrophorum* in these samples frequently differed from those on feet. Consequently, mouths and faeces are unlikely to be reservoir sites for the strains of *F. necrophorum* detected on feet. The presence of different strains of *F. necrophorum* on feet compared with mouths and faeces suggest that some strains are adapted to this site and might spread between feet.

# Chapter 6 General discussion, conclusions and future research

## 6.1 Key findings

1. The environment is not a significant reservoir of *F. necrophorum* in sheep flocks
2. Certain strains of *F. necrophorum* appear to be associated with feet and footrot
3. Diseased feet are the primary site of persistence of *F. necrophorum* strains associated with footrot
4. Healthy feet, faeces and mouths intermittently harbour strains of *F. necrophorum* associated with footrot

## 6.2 Discussion of key findings

The aim of the current study was to identify reservoir sites of *F. necrophorum* in sheep and their environment, and to understand their relevance for ovine footrot.

A key finding from this study was that *F. necrophorum* was rarely detected in the environment of sheep, overturning the belief that *F. necrophorum* is ubiquitous on sheep pasture. When detected in soil, *F. necrophorum* was transiently present and presence was likely to be due to contamination from infected sheep (Sections 3.3.3 and 3.4.3). The belief that *F. necrophorum* was able to survive in soil was based on evidence from soil microcosm experiments (Garcia *et al.*, 1971). This led to the assumption that *F. necrophorum* would be widespread on sheep pasture (Section 1.7), however, until recently no studies had tested soil from sheep pasture for *F. necrophorum*. Witcomb (2012) failed to detect *F. necrophorum* in soil samples taken from sheep pasture, and this supports the evidence from the studies presented here, and leads to the conclusion that soil is not a significant reservoir for *F. necrophorum* in sheep flocks.

The use of the MLVA typing scheme provided valuable information for understanding persistence and transmission of *F. necrophorum*. Results from Chapters 4 and 5 suggest that certain strains of *F. necrophorum* are associated with feet and involved in footrot. In contrast, other strains found in mouths and faeces, which were never detected on feet, are not linked to footrot. The strains found on feet were closely related using MLVA. It may be that the strains on feet share characteristics that make them well suited to the feet of sheep, whereas strains never found on feet may not possess the characteristics to be able to survive and persist on feet. If this suggestion were confirmed, it would significantly improve our understanding of reservoirs of *F. necrophorum* in footrot because only sites containing the strains relevant for footrot would need to be considered as reservoirs.

This is the first study to identify that footrot facilitates persistence of *F. necrophorum*. Previous studies have demonstrated that *F. necrophorum* could be detected on feet with footrot and healthy feet (Witcomb *et al.*, 2014; Frosth *et al.*, 2015; Maboni *et al.*, 2016), and that loads were higher on feet with footrot (Witcomb *et al.*, 2014; Witcomb *et al.*, 2015), as in studies presented in Chapter 3. However, results from Study B demonstrated that *F. necrophorum* was detected for up to 12 consecutive weeks on feet with footrot, with only transient detection on healthy feet (Section 3.4.3.4). Additionally, when *F. necrophorum* faded out from mouths and faeces (Section 3.4.3.2), feet with footrot were the only site where a population of *F. necrophorum* persisted, highlighting the importance of footrot for maintaining a population of *F. necrophorum* within this flock.

Although feet with footrot were the primary site of persistence for the strains of *F. necrophorum* involved in footrot in the studies presented here, these strains were intermittently present on healthy feet, in mouths and in faeces (Sections 4.4.3 and 5.3.2), suggesting that these sites could be transient reservoir sites of *F. necrophorum* in footrot. It is possible that these sites could be relevant for *F. necrophorum* persistence within a flock in the absence of footrot lesions, but further investigation would be necessary to

confirm this. Mouths and faeces did represent a persistent reservoir for strains of *F. necrophorum* not found on feet (Section 4.4.3 and 5.3.2), and a complex community of *F. necrophorum* persisted in the mouths of sheep in Study A. Further work is needed to understand farm level differences in the presence of these communities in the mouths of sheep.

This study provided evidence that footrot facilitated growth of *F. necrophorum* on feet (Section 3.4.5), and the resultant high loads of *F. necrophorum* on feet with footrot (Section 3.4.3.3) may make these feet an important source of *F. necrophorum* for transmission between feet and sheep within a flock. However, for other bacterial pathogens, there is evidence that shedding of high loads does not necessary lead to a proportionate effect on transmission (Spencer *et al.*, 2015; Slater *et al.*, 2016), and therefore further investigation would be needed to determine the role of footrot for within-flock transmission of *F. necrophorum*.

The findings discussed so far refer to persistence and transmission of *F. necrophorum* within a sheep flock. In the UK, sheep farmers frequently buy in new stock from other farms, and therefore transmission of *F. necrophorum* between flocks is also possible. Assuming farmers practice good biosecurity, only healthy sheep should be introduced to a flock and therefore feet with footrot will not be a significant source of *F. necrophorum*. However, the evidence from the studies presented here highlights that these healthy sheep may be transiently carrying strains of *F. necrophorum* capable of causing footrot, either in the mouth, on healthy feet or in faeces (Section 4.4.3 and 5.3.2). These sites may therefore be significant for transmission of *F. necrophorum* between flocks.

The findings from the current study improve our understanding of *F. necrophorum* in ovine footrot as described above, and may also be relevant to other diseases. The findings regarding strain variation between sites may be relevant when considering reservoir sites and transmission patterns for other necrobacilloses. For example, in pharyngotonsilitis in

humans it has been recently suggested that *F. necrophorum* may be transmitted between individuals (Section 1.4.1), and the MLVA scheme developed here could be valuable in determining whether the same strains are present in individuals with a history of close contact, which would be suggestive of transmission. The findings from the current study regarding the potential role of footrot in persistence and transmission of *F. necrophorum* demonstrate one method by which an opportunistic pathogen may maintain itself within a host population. Other opportunists may share this strategy, and therefore this study may contribute to our understanding of the epidemiology of a number of bacterial pathogens.

## 6.3 Limitations of the current study

The low prevalence of footrot in Study B was valuable for studying *F. necrophorum* persistence as previously highlighted, however, the lack of diversity of lesion scores and disease duration made it difficult to determine associations between *F. necrophorum* detection and footrot severity or chronicity (hypothesis 3).

The data for *D. nodosus* illustrated differences in sites of persistence for the two species, with *D. nodosus* being rarely detected in faeces and mouths, but more frequently detected in the environment. Determining associations between load of *D. nodosus* and *F. necrophorum* (hypothesis 4) was therefore only relevant for feet.

Samples that were positive for *F. necrophorum* with lower loads could not be analysed using the MLVA typing scheme, and this therefore limited the conclusions that could be drawn regarding persistence and transmission of strains of *F. necrophorum*. It is also possible that this biased the analysis by only representing the higher load samples.

The findings regarding climate are specific to this study, and whilst these may give some indication of the effect of climate on *F. necrophorum* persistence and transmission, they cannot be used to predict how *F. necrophorum* will behave in other situations.

## 6.4 Conclusions

This thesis has contributed to the understanding of persistence of *F. necrophorum* in reservoirs in sheep and their environment. In addition, an MLVA typing scheme was developed and validated for *F. necrophorum*.

Contrary to previous dogma, the environment was not a significant site of persistence for *F. necrophorum,* and soil is likely to be a temporary 'fomite' to facilitate transmission between hosts. The feet of sheep were the primary site of persistence for *F. necrophorum*, with footrot facilitating persistence. This study provided the first evidence that *F. necrophorum* is shed in sheep faeces, and both faeces and the mouths of sheep may be transient reservoirs for *F. necrophorum* in footrot. There was evidence that *F. necrophorum* formed part of a microbial community in the mouths of sheep, but the strains in this community were frequently different to those involved in footrot.

## 6.5 Future work

Future work should focus on the role of footrot in the transmission of *F. necrophorum* between sheep. For other pathogens it has been demonstrated that "super shedders" are not necessarily "super spreaders" (Slater *et al.*, 2016), and therefore this would be interesting to investigate for *F. necrophorum*. It would also be useful to examine the effects of temperature and rainfall on transmission between feet under different climatic conditions than found in the current study.

The role of carriers of *F. necrophorum* at sites other than feet with footrot in persistence would be an interesting area for future study. It is possible that these sites are relevant when footrot is completely absent from a flock, or for the transfer of *F. necrophorum* between flocks. There is evidence that *F. necrophorum* can be detected in flocks without footrot (Monaghan, unpublished data) and these flocks would therefore be useful for further study.

The MLVA typing scheme revealed differences in the communities and strains of *F. necrophorum* present on the feet and in the mouths of sheep. Further research using sequencing methods to analyse individual isolates representative of the strains found on feet and in mouths would improve our understanding of the similarities and differences between the strains identified. It would also provide information as to whether the differences between strains are related to differences in pathogenicity, or to differences in their ability to survive under different environmental conditions.

Optimisation of the MLVA typing scheme would also be valuable to allow a larger number of samples with lower loads to be analysed. This could initially involve making alterations to the Fn13 primers to improve their sensitivity so that they were more comparable to the other two loci in the scheme.

There was evidence from Study B that farm management practices may influence *F. necrophorum* epidemiology as changes in detection frequency and dominant strain type were observed following separation of healthy sheep onto un-grazed pasture. In addition, there was evidence for the potential role of climate and stocking density in environmental transmission and survival of *F. necrophorum*. Historically, management practices to reduce pathogen transmission have focused on *D. nodosus* because of assumptions regarding the widespread distribution of *F. necrophorum* in the environment. In light of the current findings, it would be valuable to investigate the effect of management practices such as quarantine, stocking density, pasture management and prompt treatment on the prevalence and diversity of *F. necrophorum*.

# References


Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., Gamonal, J. & Diaz, P. I. (2013) The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *Isme Journal*, 7 (5): 1016-1025.

Aghamiri, S. M., Haghkhah, M., Ahmadi, M. R. & Gheisari, H. R. (2014) Development of a multiplex PCR for the identification of major pathogenic bacteria of post-partum endometritis in dairy cows. *Reproduction in Domestic Animals*, 49 (2): 233-238.

Aliyu, S. H., Marriott, R. K., Curran, M. D., Parmar, S., Bentley, N., Brown, N. M., Brazier, J. S. & Ludlam, H. (2004) Real-time PCR investigation into the importance of *Fusobacterium necrophorum* as a cause of acute pharyngitis in general practice. *Journal of Medical Microbiology*, 53 (10): 1029-1035.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, 215 (3): 403-410.

Amoako, K. K., Goto, Y., Misawa, N., Xu, D. L. & Shinjo, T. (1998) The erythrocyte receptor for *Fusobacterium necrophorum* hemolysin: phosphatidylcholine as a possible candidate. *FEMS Microbiology Letters*, 168 (1): 65-70.

Amoako, K. K., Goto, Y. & Shinjo, T. (1994) Studies on the factors affecting the hemolytic activity of *Fusobacterium necrophorum*. *Veterinary Microbiology*, 41 (1-2): 11-18.

Amoako, K. K., Goto, Y., Xu, D. L. & Shinjo, T. (1996) The effects of physical and chemical agents on the secretion and stability of a *Fusobacterium necrophorum* hemolysin. *Veterinary Microbiology*, 51 (1–2): 115-124.

Antiabong, J. F., Boardman, W., Smith, I., Brown, M. H., Ball, A. S. & Goodman, A. E. (2013a) "Cycliplex PCR" confirmation of *Fusobacterium necrophorum* isolates from captive wallabies: a rapid and accurate approach. *Anaerobe*, 19: 44-49.

Antiabong, J. F., Boardman, W., Smith, I., Brown, M. H., Ball, A. S. & Goodman, A. E. (2013b) A molecular survey of a captive wallaby population for periodontopathogens and the co-incidence of *Fusobacterium necrophorum* subspecies *necrophorum* with periodontal diseases. *Veterinary Microbiology*, 163 (3-4): 335-343.

Anttila, J., Kaitala, V., Laakso, J. & Ruokolainen, L. (2015) Environmental variation generates environmental opportunist pathogen outbreaks. *PLoS One*, 10 (12): e0145511.

Atia, J., Monaghan, E., Kaler, J., Purdy, K., Green, L. & Keeling, M. (2017) Mathematical modeling of ovine footrot in the UK: the effect of *Dichelobacter nodosus* and *Fusobacterium necrophorum* on the disease dynamics. *Epidemics*, https://doi.org/10.1016/j.epidem.2017.04.001.

Bartholomew, J. W. & Mittwer, T. (1952) The Gram stain. *Bacteriological Reviews*, 16 (1): 1-29.

Bartlett, J. G. & Gorbach, S. L. (1976) Anaerobic infections of head and neck. *Otolaryngologic Clinics of North America*, 9 (3): 655-678.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67 (1): 1-48.

Bennett, G., Hickford, J., Sedcole, R. & Zhou, H. (2009) *Dichelobacter nodosus*, *Fusobacterium necrophorum* and the epidemiology of footrot. *Anaerobe*, 15 (4): 173-176.

Benson, G. (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research*, 27 (2): 573-580.

Berghaus, R. D., Thayer, S. G., Law, B. F., Mild, R. M., Hofacre, C. L. & Singer, R. S. (2013) Enumeration of *Salmonella* and *Campylobacter* spp. in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. *Applied and Environmental Microbiology*, 79 (13): 4106-4114.

Beveridge, W. I. B. (1941) Foot-rot in sheep: a transmissible disease due to infection with *Fusiformis nodosus* (n.sp.): studies on its cause, epidemiology and control. *CSIRO Australian Bulletin*, 140: 1-56.

Brauner, A., Fridman, O., Gefen, O. & Balaban, N. Q. (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews Microbiology*, 14 (5): 320-330.

Brazier, J. S., Citron, D. M. & Goldstein, E. J. C. (1991) A selective medium for *Fusobacterium* spp. *Journal of Applied Bacteriology*, 71 (4): 343-346.

Brooks, J. W., Kumar, A., Narayanan, S., Myers, S., Brown, K., Nagaraja, T. G. & Jayarao, B. M. (2014) Characterization of *Fusobacterium* isolates from the respiratory tract of white-tailed deer (*Odocoileus virginianus*). *Journal of Veterinary Diagnostic Investigation*, 26 (2): 213-220.

Brown, P. E., Christensen, O. F., Clough, H. E., Diggle, P. J., Hart, C. A., Hazel, S., Kemp, R., Leatherbarrow, A. J. H., Moore, A., Sutherst, J., Turner, J., Williams, N. J., Wright, E. J. & French, N. P. (2004) Frequency and spatial distribution of environmental *Campylobacter* spp. *Applied and Environmental Microbiology*, 70 (11): 6501-6511.

Calcutt, M. J., Foecking, M. F., Nagaraja, T. G. & Stewart, G. C. (2014) Draft genome sequence of *Fusobacterium necrophorum* subsp. *funduliforme* bovine liver abscess isolate B35. *Genome announcements*, 2 (2): e00412-00414.

Calvo-Bado, L. A., Oakley, B. B., Dowd, S. E., Green, L. E., Medley, G. F., Ul-Hassan, A., Bateman, V., Gaze, W., Witcomb, L., Grogono-Thomas, R., Kaler, J., Russell, C. L. & Wellington, E. M. H. (2011) Ovine pedomics: the first study of the ovine foot 16S rRNA-based microbiome. *The ISME Journal*, 5 (9): 1426-1437.

Clark, B. L., Stewart, D. J. & Emery, D. L. (1985) The role of *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* in the etiology of interdigital necrobacillosis in cattle. *Australian Veterinary Journal*, 62 (2): 47-49.

Clifton, R. & Green, L. E. (2016) On the treatment, control and elimination of ovine footrot: A comparative review. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 11: 053.

Cobbold, R. N., Hancock, D. D., Rice, D. H., Berg, J., Stilborn, R., Hovde, C. J. & Besser, T. E. (2007) Rectoanal junction colonization of feedlot cattle by *Escherichia coli* O157 : H7 and its association with supershedders and excretion dynamics. *Applied and Environmental Microbiology*, 73 (5): 1563-1568.

Davies, P. L., Leigh, J. A., Bradley, A. J., Archer, S. C., Emes, R. D. & Green, M. J. (2016) Molecular epidemiology of *Streptococcus uberis* clinical mastitis in dairy herds: strain heterogeneity and transmission. *Journal of Clinical Microbiology*, 54 (1): 68-74.

Defra (2011) *Sheep: Condition Scoring (PB1875)*. [online] Available from: http://adlib.everysite.co.uk/adlib/defra/content.aspx?doc=12790&id=12795 (Accessed Aug 2017).

Depiazzi, L. J., Roberts, W. D., Hawkins, C. D., Palmer, M. A., Pitman, D. R., McQuade, N. C., Jelinek, P. D., Devereuax, D. J. & Rippon, R. J. (1998) Severity and persistence of footrot in Merino sheep experimentally infected with a protease thermostable strain of *Dichelobacter nodosus* at five sites. *Australian Veterinary Journal*, 76 (1): 32-38.

Dubreuil, P. & Anderson, D. E. (2009) Chapter 54 - small ruminant infectious disease of the foot. In: Rings, D. M. eds. *Food Animal Practice (Fifth Edition)*. Saint Louis: W.B. Saunders.

Eaton, C. & Swindells, J. (2014) The significance and epidemiology of *Fusobacterium necrophorum* in sore throats. *Journal of Infection*, 69 (2): 194-196.

Edwards, J. F., Davis, D. S., Roffe, T. J., Ramiro-Ibanez, F. & Elzer, P. H. (2001) Fusobacteriosis in captive wild-caught pronghorns (*Antilocapra americana*). *Veterinary Pathology*, 38 (5): 549-552.

Egerton, J. R., Ghimire, S. C., Dhungyel, O. P., Shrestha, H. K., Joshi, H. D., Joshi, B. R., Abbott, K. A. & Kristo, C. (2002) Eradication of virulent footrot from sheep and goats in an endemic area of Nepal and an evaluation of specific vaccination. *Veterinary Record*, 151 (10): 290-295.

Egerton, J. R., Roberts, D. S. & Parsonson, I. M. (1969) The aetiology and pathogenesis of ovine foot-rot. I. A histological study of the bacterial invasion. *Journal of Comparative Pathology*, 79 (2): 207-215.

Eisenberg, T., Wolter, W., Lenz, M., Schlez, K. & Zschock, M. (2013) Boot swabs to collect environmental samples from common locations in dairy herds for *Mycobacterium avium* ssp *paratuberculosis* (MAP) detection. *Journal of Dairy Research*, 80 (4): 485-489.

Enwonwu, C. O., Falkler, W. A., Jr., Idigbe, E. O., Afolabi, B. M., Ibrahim, M., Onwujekwe, D., Savage, O. & Meeks, V. I. (1999) Pathogenesis of cancrum oris (noma): confounding interactions of malnutrition with infection. *Am J Trop Med Hyg*, 60 (2): 223-232.

Eriksen, N. H., Espersen, F., Rosdahl, V. T. & Jensen, K. (1994) Evaluation of methods for the detection of nasal carriage of *Staphylococcus aureus*. *APMIS*, 102 (6): 407-412.

Eyre, D. W., Fawley, W. N., Best, E. L., Griffiths, D., Stoesser, N. E., Crook, D. W., Peto, T. E. A., Walker, A. S. & Wilcox, M. H. (2013) Comparison of multilocus variable-number tandem-repeat analysis and whole-genome sequencing for investigation of *Clostridium difficile* transmission. *Journal of Clinical Microbiology*, 51 (12): 4141-4149.

Fay, M. P. & Shaw, P. A. (2010) Exact and asymptotic weighted logrank tests for interval censored data: the interval R package. *Journal of Statistical Software*, 36 (2): 1-34.

Forbes, A. B., Strobel, H. & Stamphoj, I. (2014) Field studies on the elimination of footrot in sheep through whole flock treatments with gamithromycin. *Veterinary Record*, 174 (6): 146.

Francisco, A. P., Bugalho, M., Ramirez, M. & Carriço, J. A. (2009) Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. *BMC Bioinformatics*, 10 (1): 1-15.

Francisco, A. P., Vaz, C., Monteiro, P. T., Melo-Cristino, J., Ramirez, M. & Carriço, J. A. (2012) PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. *BMC Bioinformatics*, 13 (1): 1-10.

Frosth, S., Koenig, U., Nyman, A.-K., Pringle, M. & Aspan, A. (2015) Characterisation of *Dichelobacter nodosus* and detection of *Fusobacterium necrophorum* and *Treponema* spp. in sheep with different clinical manifestations of footrot. *Veterinary Microbiology*, 179 (1-2): 82-90.

Gao, Z., Perez-Perez, G. I., Chen, Y. & Blaser, M. J. (2010) Quantitation of major human cutaneous bacterial and fungal populations. *Journal of Clinical Microbiology*, 48 (10): 3575-3581.

Garcia, G. G., Goto, Y. & Shinjo, T. (2000) Endotoxin-triggered haematological interactions in *Fusobacterium necrophorum* infections. *Microbios*, 102 (401): 39-44.

Garcia, M. M., Neil, D. H. & McKay, K. A. (1971) Application of immunofluorescence to studies on the ecology of *Sphaerophorus necrophorus*. *Applied Microbiology*, 21 (5): 809-814.

Gibbons, J. F., Markey, B. K., Jahns, H., Boland, F., Abbott, Y., Burns, A., Egan, J., Fanning, S., Gutierrez, M. & Leonard, F. C. (2013) Investigation of the persistence and transmission of MRSA CC 5 in pigs following intra-nasal inoculation. *Veterinary Microbiology*, 162 (2-4): 771-778.

Goddard, P., Waterhouse, T., Dwyer, C. & Stott, A. (2006) The perception of the welfare of sheep in extensive systems. *Small Ruminant Research*, 62 (3): 215-225.

Gomes, B., Pinheiro, E., Gadê‐Neto, C., Sousa, E., Ferraz, C., Zaia, A., Teixeira, F. & Souza‐Filho, F. (2004) Microbiological examination of infected dental root canals. *Oral microbiology and immunology*, 19 (2): 71-76.

Gomez, A. & Nelson, K. E. (2017) The oral microbiome of children: development, disease, and implications beyond oral health. *Microbial Ecology*, 73 (2): 492-503.

Graham, N. P. & Egerton, J. R. (1968) Pathogenesis of ovine foot-rot: the role of some environmental factors. *Australian Veterinary Journal*, 44 (5): 235-240.

Greber, D., Bearth, G., Luchinger, R., Schuepbach-Regula, G. & Steiner, A. (2016) Elimination of virulent strains (aprV2) of *Dichelobacter nodosus* from feet of 28 Swiss sheep flocks: A proof of concept study. *Veterinary Journal*, 216: 25-32.

Green, L. E. & George, T. R. N. (2008) Assessment of current knowledge of footrot in sheep with particular reference to *Dichelobacter nodosus* and implications for elimination or control strategies for sheep in Great Britain. *The Veterinary Journal*, 175 (2): 173-180.

Green, L. E., Wassink, G. J., Grogono-Thomas, R., Moore, L. J. & Medley, G. F. (2007) Looking after the individual to reduce disease in the flock: A binomial mixed effects model investigating the impact of individual sheep management of footrot and interdigital dermatitis in a prospective longitudinal study on one farm. *Preventive Veterinary Medicine*, 78 (2): 172-178.

Grogono-Thomas, R. & Johnston, A. (1997) *A study of ovine lameness. MAFF Final Report MAFF Open Contract OC59 45K*. London: DEFRA Publications.

Grundmann, H., Hori, S. & Tanner, G. (2001) Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. *Journal of Clinical Microbiology*, 39 (11): 4190-4192.

Gurung, R. B., Dhungyel, O. P., Tshering, P. & Egerton, J. R. (2006) The use of an autogenous *Dichelobacter nodosus* vaccine to eliminate clinical signs of virulent footrot in a sheep flock in Bhutan. *Veterinary Journal*, 172 (2): 356-363.

Hackett, S. J., Guiver, M., Marsh, J., Sills, J. A., Thomson, A. P. J., Kaczmarski, E. B. & Hart, C. A. (2002) Meningococcal bacterial DNA load at presentation correlates with disease severity. *Archives of Disease in Childhood*, 86 (1): 44-46.

Hajishengallis, G., Darveau, R. P. & Curtis, M. A. (2012) The keystone pathogen hypothesis. *Nature reviews. Microbiology*, 10 (10): 717-725.

Hajishengallis, G., Liang, S., Payne, M. A., Hashim, A., Jotwani, R., Eskan, M. A., McIntosh, M. L., Alsam, A., Kirkwood, K. L., Lambris, J. D., Darveau, R. P. & Curtis, M. A. (2011) Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe*, 10 (5): 497-506.

Halkilahti, J., Haukka, K. & Siitonen, A. (2013) Genotyping of outbreak-associated and sporadic *Yersinia pseudotuberculosis* strains by novel multilocus variable-number tandem repeat analysis (MLVA). *Journal of Microbiological Methods*, 95 (2): 245-250.

Hall, V., Duerden, B. I., Magee, J. T., Ryley, H. C. & Brazier, J. S. (1997) A comparative study of *Fusobacterium necrophorum* strains from human and animal sources by phenotypic reactions, pyrolysis mass spectrometry and SDS-PAGE. *Journal of Medical Microbiology*, 46 (10): 865-871.

Handeland, K., Boye, M., Bergsjo, B., Bondal, H., Isaksen, K. & Agerholm, J. S. (2010) Digital necrobacillosis in Norwegian wild tundra reindeer (*Rangifer tarandus tarandus*). *Journal of Comparative Pathology*, 143 (1): 29-38.

Hill, A. E., Dhungyel, O. P. & Whittington, R. J. (2010) Diagnostic sampling strategies for virulent ovine footrot: Simulating detection of *Dichelobacter nodosus* serogroups for bivalent vaccine formulation. *Preventive Veterinary Medicine*, 95 (1-2): 127-136.

Holm, K., Bank, S., Nielsen, H., Kristensen, L. H., Prag, J. & Jensen, A. (2016) The role of *Fusobacterium necrophorum* in pharyngotonsillitis - A review. *Anaerobe*, 42: 89-97.

Holt, R. D. (1993) Ecology at the mesoscale: the influence of regional processes on local communities. In: Ricklefs, R. E. & Schluter, D. eds. *Species diversity in ecological communities: Historical and geographical perspectives*. London, England: University of Chicago Press.

Horose, M., Kiyoyama, H., Ogawa, H. & Shinjo, T. (1992) Aggregation of bovine platelets by *Fusobacterium necrophorum*. *Veterinary Microbiology*, 32 (3-4): 343-350.

Hunter, P. R. & Gaston, M. A. (1988) Numerical index of the discriminatory ability of typing systems - an application of Simpson's Index of Diversity. *Journal of Clinical Microbiology*, 26 (11): 2465-2466.

Jacinto, R. C., Montagner, F., Signoretti, F. G. C., Almeida, G. C. & Gomes, B. (2008) Frequency, microbial interactions, and antimicrobial susceptibility of *Fusobacterium nucleatum* and *Fusobacterium necrophorum* isolated from primary endodontic infections. *Journal of Endodontics*, 34 (12): 1451-1456.

Jensen, A., Hansen, T. M., Bank, S., Kristensen, L. H. & Prag, J. (2015) *Fusobacterium necrophorum* tonsillitis: an important cause of tonsillitis in adolescents and young adults. *Clinical Microbiology and Infection*, 21 (3): 266.e261-266.e263.

Jensen, A., Kristensen, L. H. & Prag, J. (2007) Detection of *Fusobacterium necrophorum* subsp. *funduliforme* in tonsillitis in young adults by real-time PCR. *Clinical Microbiology and Infection*, 13 (7): 695-701.

Kaler, J., Daniels, S. L. S., Wright, J. L. & Green, L. E. (2010a) Randomized clinical trial of long-acting oxytetracycline, foot trimming, and flunixine meglumine on time to recovery in sheep with footrot. *Journal of Veterinary Internal Medicine*, 24 (2): 420-425.

Kaler, J. & Green, L. E. (2008) Naming and recognition of six foot lesions of sheep using written and pictorial information: A study of 809 English sheep farmers. *Preventive Veterinary Medicine*, 83 (1): 52-64.

Kaler, J. & Green, L. E. (2009) Farmers' practices and factors associated with the prevalence of all lameness and lameness attributed to interdigital dermatitis and footrot in sheep flocks in England in 2004. *Preventive Veterinary Medicine*, 92 (1-2): 52-59.

Kaler, J., Medley, G. F., Grogono-Thomas, R., Wellington, E. M. H., Calvo-Bado, L. A., Wassink, G. J., King, E. M., Moore, L. J., Russell, C. & Green, L. E. (2010b) Factors associated with changes of state of foot conformation and lameness in a flock of sheep. *Preventive Veterinary Medicine*, 97 (3-4): 237-244.

Kennan, R. M., Han, X. Y., Porter, C. J. & Rood, J. I. (2011) The pathogenesis of ovine footrot. *Veterinary Microbiology*, 153 (1-2): 59-66.

Kersh, G. J., Fitzpatrick, K. A., Self, J. S., Priestley, R. A., Kelly, A. J., Lash, R. R., Marsden-Haug, N., Nett, R. J., Bjork, A., Massung, R. F. & Anderson, A. D. (2013) Presence and persistence of *Coxiella burnetii* in the environments of goat farms associated with a Q fever outbreak. *Applied and Environmental Microbiology*, 79 (5): 1697-1703.

Krämer, A., Akmatov, M. & Kretzschmar, M. (2010) Principles of infectious disease epidemiology. In: Krämer, A., Kretzschmar, M. & Krickeberg, K. eds. *Modern Infectious Disease Epidemiology: Concepts, Methods, Mathematical Models, and Public Health*. New York, NY: Springer New York.

Kumar, A., Gart, E., Nagaraja, T. G. & Narayanan, S. (2013) Adhesion of *Fusobacterium necrophorum* to bovine endothelial cells is mediated by outer membrane proteins. *Veterinary Microbiology*, 162 (2-4): 813-818.

Kuppalli, K., Livorsi, D., Talati, N. J. & Osborn, M. (2012) Lemierre's syndrome due to *Fusobacterium necrophorum*. *The Lancet Infectious Diseases*, 12 (10): 808-815.

Langworth, B. F. (1977) *Fusobacterium necrophorum:* its characteristics and role as an animal pathogen. *Bacteriological Reviews*, 41 (2): 373-390.

Lechtenberg, K. F., Nagaraja, T. G., Leipold, H. W. & Chengappa, M. M. (1988) Bacteriologic and histologic studies of hepatic abscesses in cattle. *American Journal of Veterinary Research*, 49 (1): 58-62.

LeJeune, J. T., Besser, T. E., Rice, D. H., Berg, J. L., Stilborn, R. P. & Hancock, D. D. (2004) Longitudinal study of fecal shedding of *Escherichia coli* O157 : H7 in feedlot cattle: Predominance and persistence of specific clonal types despite massive cattle population turnover. *Applied and Environmental Microbiology*, 70 (1): 377-384.

Lemierre, A. (1936) On certain speticaemias due to anaerobic organisms. *Lancet*, ii: 701-703.

Ley, S. J., Waterman, A. E. & Livingston, A. (1995) A field study of the effect of lameness on mechanical nociceptive thresholds in sheep. *Veterinary Record*, 137 (4): 85-87.

Liebana, E., Smith, R. P., Batchelor, M., McLaren, I., Cassar, C., Clifton-Hadley, F. A. & Paiba, G. A. (2005) Persistence of *Escherichia coli* O157 isolates on bovine farms in England and Wales. *Journal of Clinical Microbiology*, 43 (2): 898-902.

Loesche, W. J. (1976) Chemotherapy of dental plaque infections. *Oral Sci Rev*, 9: 65-107.

Ludlam, H., Howard, J., Kingston, B., Donachie, L., Foulkes, J., Guha, S. & Curran, M. D. (2009) Epidemiology of pharyngeal carriage of *Fusobacterium necrophorum*. *Journal of Medical Microbiology*, 58 (9): 1264-1265.

Maboni, G., Frosth, S., Aspan, A. & Totemeyer, S. (2016) Ovine footrot: new insights into bacterial colonisation. *Veterinary Record*, 179 (9): 228.

Marin, C. & Lainez, M. (2009) *Salmonella* detection in feces during broiler rearing and after live transport to the slaughterhouse. *Poultry Science*, 88 (9): 1999-2005.

Marsh, H. & Tunnicliff, E. A. (1934) Experimental studies of foot-rot in sheep. *Montana Agricultural Experiment Station Bulletin*, 285: 3-16.

Marshall, D. J., Walker, R. I., Cullis, B. R. & Luff, M. F. (1991) The effect of footrot on body weight and wool growth of sheep. *Australian Veterinary Journal*, 68 (2): 45-49.

McCourtie, J., Poxton, I. R., Brown, R., Whittaker, C. R., Spence, J. A. & Aitchison, G. U. (1990) A longitudinal study of the cultivable subgingival anaerobic bacteria isolated from sheep during the development of broken mouth periodontitis. *Journal of Medical Microbiology*, 31 (4): 275-283.

Mehraj, J., Witte, W., Akmatov, M. K., Layer, F., Werner, G. & Krause, G. (2016) Epidemiology of *Staphylococcus aureus* nasal carriage patterns in the community. In: Stadler, M. & Dersch, P. eds. *How to Overcome the Antibiotic Crisis : Facts, Challenges, Technologies and Future Perspectives*. Cham: Springer International Publishing.

Mezal, E. H., Sabol, A., Khan, M. A., Ali, N., Stefanova, R. & Khan, A. A. (2014) Isolation and molecular characterization of *Salmonella enterica* serovar Enteritidis from poultry house and clinical samples during 2010. *Food Microbiology*, 38: 67-74.

Mills, K., McClenaughan, P., Morton, A., Alley, D., Lievaart, J., Windsor, P. A. & Egerton, J. R. (2012) Effect on time in quarantine of the choice of program for eradication of footrot from 196 sheep flocks in southern New South Wales. *Australian Veterinary Journal*, 90 (1-2): 14-19.

Mohler, J. R. & Washburn, H. J. (1904) *Foot-rot of sheep: its nature, cause, and treatment*. US Department of Agriculture, Bureau of Animal Industry.

Monrad, J., Kassuku, A. A., Nansen, P. & Willeberg, P. (1983) An epidemiological study of foot rot in pastured cattle. *Acta Veterinaria Scandinavica*, 24 (4): 403-417.

Moore, L. J., Woodward, M. J. & Grogono-Thomas, R. (2005) The occurrence of treponemes in contagious ovine digital dermatitis and the characterisation of associated *Dichelobacter nodosus*. *Veterinary Microbiology*, 111 (3-4): 199-209.

Moore, W. E., Holdeman, L. V., Smibert, R. M., Hash, D. E., Burmeister, J. A. & Ranney, R. R. (1982) Bacteriology of severe periodontitis in young adult humans. *Infect Immun*, 38 (3): 1137-1148.

Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T. L., Agarwala, R. & Schaffer, A. A. (2008) Database indexing for production MegaBLAST searches. *Bioinformatics*, 24 (16): 1757-1764.

Mork, T., Kvitle, B. & Jorgensen, H. J. (2012) Reservoirs of *Staphylococcus aureus* in meat sheep and dairy cattle. *Veterinary Microbiology*, 155 (1): 81-87.

Nagaraja, T. G. & Chengappa, M. M. (1998) Liver abscesses in feedlot cattle: a review. *Journal of Animal Science*, 76 (1): 287-298.

Nagaraja, T. G., Narayanan, S. K., Stewart, G. C. & Chengappa, M. M. (2005) *Fusobacterium necrophorum* infections in animals: pathogenesis and pathogenic mechanisms. *Anaerobe*, 11 (4): 239-246.

Narayanan, S., Nagaraja, T. G., Okwumabua, O., Staats, J., Chengappa, M. M. & Oberst, R. D. (1997) Ribotyping to compare *Fusobacterium necrophorum* isolates from bovine liver abscesses, ruminal walls, and ruminal contents. *Applied and Environmental Microbiology*, 63 (12): 4671-4678.

Narayanan, S., Stewart, G. C., Chengappa, M. M., Willard, L., Shuman, W., Wilkerson, M. & Nagaraja, T. G. (2002) *Fusobacterium necrophorum* leukotoxin induces activation and apoptosis of bovine leukocytes. *Infection and Immunity*, 70 (8): 4609-4620.

Narongwanichgarn, W., Misawa, N., Jin, J. H., Amoako, K. K., Kawaguchi, E., Shinjo, T., Haga, T. & Goto, Y. (2003) Specific detection and differentiation of two subspecies of *Fusobacterium necrophorum* by PCR. *Veterinary Microbiology*, 91 (2-3): 183-195.

Naylor, R. D., Martin, P. K., Jones, J. R. & Burnell, M. C. (1998) Isolation of spirochaetes from an incident of severe virulent ovine footrot. *Veterinary Record*, 143 (25): 690.

Nieuwhof, G. J. & Bishop, S. C. (2005) Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. *Animal Science*, 81: 23-29.

Nieuwhof, G. J., Bishop, S. C., Hill, W. G. & Raadsma, H. W. (2008) The effect of footrot on weight gain in sheep. *Animal*, 2 (10): 1427-1436.

Nightingale, K. K., Schukken, Y. H., Nightingale, C. R., Fortes, E. D., Ho, A. J., Her, Z., Grohn, Y. T., McDonough, P. L. & Wiedmann, M. (2004) Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. *Applied and Environmental Microbiology*, 70 (8): 4458-4467.

Nilsson, A. C., Bjorkman, P., Welinder-Olsson, C., Widell, A. & Persson, K. (2010) Clinical severity of *Mycoplasma pneumoniae* (MP) infection is associated with bacterial load in oropharyngeal secretions but not with MP genotype. *BMC Infectious Diseases*, 10.

Nobbs, A. H., Dymock, D. & Jenkinson, H. F. (2013) Microbiota of the mouth: a blessing or a curse? In: eds. *The Human Microbiota*. John Wiley & Sons, Inc.

Okamoto, K., Kanoe, M., Inoue, M., Watanabe, T. & Inoue, T. (2005) Dermotoxic activity of a collagenolytic cell wall component from *Fusobacterium necrophorum* subsp. *necrophorum*. *Veterinary Journal*, 169 (2): 308-310.

Omisakin, F., MacRae, M., Ogden, I. D. & Strachan, N. J. C. (2003) Concentration and prevalence of *Escherichia coli* O157 in cattle feces at slaughter. *Applied and Environmental Microbiology*, 69 (5): 2444-2447.

Panciera, R. J., Perino, L. J., Baldwin, C. A., Morton, R. J. & Swanson, J. E. (1989) Observations on calf diphtheria in the commercial feedlot. *Agri-Practice*, 10 (5): 12-17.

Patra, P. & Klumpp, S. (2013) Population dynamics of bacterial persistence. *PLoS One*, 8 (5): 1-9.

Petrie, A. & Watson, P. (2013) *Statistics for Veterinary and Animal Science*. Third edn. Chichester, UK: Wiley-Blackwell.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team (2016) nlme: linear and nonlinear mixed effects models. http://cran.r-project.org/package=nlme.

Purdy, K. J. (2005) Nucleic acid recovery from complex environmental samples. *Methods in Enzymology*, 397: 271-292.

R Development Core Team (2008) R: A language and environment for statistical computing. http://www.r-project.org/.

Raizman, E. A., Wells, S. J., Godden, S. M., Bey, R. F., Oakes, M. J., Bentley, D. C. & Olsen, K. E. (2004) The distribution of *Mycobacterium avium* ssp *paratuberculosis* in the environment surrounding Minnesota dairy farms. *Journal of Dairy Science*, 87 (9): 2959-2966.

Renter, D. G., Sargeant, J. M., Oberst, R. D. & Samadpour, M. (2003) Diversity, frequency, and persistence of *Escherichia coli* O157 strains from range cattle environments. *Applied and Environmental Microbiology*, 69 (1): 542-547.

Rice, D. H., Sheng, H. Q. Q., Wynia, S. A. & Hovde, C. J. (2003) Rectoanal mucosal swab culture is more sensitive than fecal culture and distinguishes *Escherichia coli* O157 : H7-colonized cattle and those transiently shedding the same organism. *Journal of Clinical Microbiology*, 41 (11): 4924-4929.

Ridler, A., Wilson, D. & Nixon, N. (2009) *Effect of environmental and climatic conditions on footrot in sheep in the UK*. IN: Proceedings of the 7th International Sheep Veterinary Congress. Stavanger, Norway. 104-105.

Riordan, T. (2007) Human infection with *Fusobacterium necrophorum* (Necrobacillosis), with a focus on Lemierre's syndrome. *Clinical Microbiology Reviews*, 20 (4): 622-659.

Roberts, D. S. (1967) The pathogenic synergy of *Fusiformis necrophorus* and *Corynebacterium pyogenes*. I. Influence of the leucocidal exotoxin of *F. necrophorus*. *British journal of experimental pathology*, 48 (6): 665-673.

Roberts, D. S. & Egerton, J. R. (1969) The aetiology and pathogenesis of ovine foot-rot. II. The pathogenic association of *Fusiformis nodosus* and *Fusiformis necrophorus*. *Journal of Comparative Pathology*, 79 (2): 217-227.

Rogers, M. B., Brower-Sinning, R., Firek, B., Zhong, D. & Morowitz, M. J. (2016) Acute appendicitis in children is associated with a local expansion of *Fusobacteria*. *Clinical Infectious Diseases*, 63 (1): 71-78.

Ruder, C. A., Sasser, R. G., Williams, R. J., Ely, J. K., Bull, R. C. & Butler, J. E. (1981) Uterine infections in the postpartum cow 2. Possible synergistic effect of *Fusobacterium necrophorum* and *Corynebacterium pyogenes*. *Theriogenology*, 15 (6): 573-580.

Russell, C. L., Smith, E. M., Calvo-Bado, L. A., Green, L. E., Wellington, E. M., Medley, G. F., Moore, L. J. & Grogono-Thomas, R. (2013a) Multiple locus VNTR analysis highlights that geographical clustering and distribution of *Dichelobacter nodosus*, the causal agent of footrot in sheep, correlates with inter-country movements. *Infection, Genetics and Evolution*, 22: 273-279.

Russell, V. N. L., Green, L. E., Bishop, S. C. & Medley, G. F. (2013b) The interaction of host genetics and disease processes in chronic livestock disease: A simulation model of ovine footrot. *Preventive Veterinary Medicine*, 108 (4): 294-303.

Sanchez, S., Martinez, R., Garcia, A., Blanco, J., Blanco, J. E., Blanco, M., Dahbi, G., Lopez, C., Mora, A., Rey, J. & Alonso, J. M. (2009) Longitudinal study of shiga toxin-producing *Escherichia coli* shedding in sheep feces: persistence of specific clones in sheep flocks. *Applied and Environmental Microbiology*, 75 (6): 1769-1773.

Selenska, S. & Klingmüller, W. (1991) DNA recovery and direct detection of Tn5 sequences from soil. *Letters in Applied Microbiology*, 13 (1): 21-24.

Shah, H. N., Olsen, I., Bernard, K., Finegold, S. M., Gharbia, S. & Gupta, R. S. (2009) Approaches to the study of the systematics of anaerobic, Gram-negative, non-sporeforming rods: Current status and perspectives. *Anaerobe*, 15 (5): 179-194.

Shere, J. A., Bartlett, K. J. & Kaspar, C. W. (1998) Longitudinal study of *Escherichia coli* O157 : H7 dissemination on four dairy farms in Wisconsin. *Applied and Environmental Microbiology*, 64 (4): 1390-1399.

Shinjo, T., Fujisawa, T. & Mitsuoka, T. (1991) Proposal of two subspecies of *Fusobacterium necrophorum* (Flugge) Moore and Holdeman: *Fusobacterium necrophorum* subsp. *necrophorum* subsp. nov., nom. rev. (ex Flugge 1886), and *Fusobacterium necrophorum* subsp. *funduliforme* subsp. nov., nom. rev. (ex Halle 1898). *International Journal of Systematic Bacteriology*, 41 (3): 395-397.

Shinjo, T. & Kiyoyama, H. (1986) Pathogenicity of a non-hemagglutinating mutant strain of *Fusobacterum necrophorum* biovar A in mice. *Japanese Journal of Veterinary Science*, 48 (3): 523-527.

Slater, N., Mitchell, R. M., Whitlock, R. H., Fyock, T., Pradhan, A. K., Knupfer, E., Schukken, Y. H. & Louzoun, Y. (2016) Impact of the shedding level on transmission of persistent infections in *Mycobacterium avium* subspecies *paratuberculosis* (MAP). *Veterinary Research*, 47: 38.

Smith, E. M., Green, O. D. J., Calvo-Bado, L. A., Witcomb, L. A., Grogono-Thomas, R., Russell, C. L., Brown, J. C., Medley, G. F., KilBride, A. L., Wellington, E. M. H. & Green, L. E. (2014) Dynamics and impact of footrot and climate on hoof horn length in 50 ewes from one farm over a period of 10 months. *Veterinary Journal*, 201 (3): 295-301.

Spencer, S. E. F., Besser, T. E., Cobbold, R. N. & French, N. P. (2015) 'Super' or just 'above average'? Supershedders and the transmission of *Escherichia coli* 0157:H7 among feedlot cattle. *Journal of the Royal Society Interface*, 12: 0446.

Sullivan, L. E., Clegg, S. R., Angell, J. W., Newbrook, K., Blowey, R. W., Carter, S. D., Bell, J., Duncan, J. S., Grove-White, D. H., Murray, R. D. & Evans, N. J. (2015) High-level association of bovine digital dermatitis *Treponema* spp. with contagious ovine digital dermatitis lesions and presence of *Fusobacterium necrophorum* and *Dichelobacter nodosus*. *Journal of Clinical Microbiology*, 53 (5): 1628-1638.

Takeuchi, S., Nakajima, Y. & Hashimoto, K. (1983) Pathogenic synergism of *Fusobacterium necrophorum* and other bacteria in formation of liver abscess in BALB/c mice. *Japanese Journal of Veterinary Science*, 45 (6): 775-781.

Tan, Z. L., Nagaraja, T. G. & Chengappa, M. M. (1994) Selective enumeration of *Fusobacterium necrophorum* from the bovine rumen. *Applied and Environmental Microbiology*, 60 (4): 1387-1389.

Tan, Z. L., Nagaraja, T. G. & Chengappa, M. M. (1996) *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanism and control measures. *Veterinary Research Communications*, 20 (2): 113-140.

Theilade, E. (1986) The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J Clin Periodontol*, 13 (10): 905-911.

Underwood, W. J., Blauwiekel, R., Delano, M. L., Gillesby, R., Mischler, S. A. & Schoell, A. (2015) Chapter 15 - Biology and diseases of ruminants (sheep, goats, and cattle). In: Anderson, L. C., Otto, G. M., Pritchett-Corning, K. R. & Whary, M. T. eds. *Laboratory Animal Medicine (Third Edition)*. Boston: Academic Press.

van Belkum, A., Tassios, P. T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N. K., Fussing, V., Green, J., Feil, E., Gerner-Smidt, P., Brisse, S., Struelens, M., Escmid & Esgem (2007) Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clinical Microbiology and Infection*, 13: 1-46.

Van, T. T., Cox, L. M., Cox, M. E. & Bard, J. D. (2017) Prevalence of *Fusobacterium necrophorum* in children presenting with pharyngitis. *Journal of Clinical Microbiology*, 55 (4): 1147-1153.

Verdier-Metz, I., Gagne, G., Bornes, S., Monsallier, F., Veisseire, P., Delbès-Paus, C. & Montel, M.-C. (2012) Cow teat skin, a potential source of diverse microbial populations for cheese production. *Applied and Environmental Microbiology*, 78 (2): 326-333.

Verhoeven, P. O., Grattard, F., Carricajo, A., Lucht, F., Cazorla, C., Garraud, O., Pozzetto, B. & Berthelot, P. (2012) Quantification by real-time PCR assay of *Staphylococcus aureus* load: a useful tool for rapidly identifying persistent nasal carriers. *Journal of Clinical Microbiology*, 50 (6): 2063-2065.

Vranckx, K., Maes, D., Calus, D., Villarreal, I., Pasmans, F. & Haesebrouck, F. (2011) Multiple-locus variable-number tandem-repeat analysis is a suitable tool for differentiation of *Mycoplasma hyopneumoniae* strains without cultivation. *Journal of Clinical Microbiology*, 49 (5): 2020-2023.

Wada, T., Maeda, S., Hase, A. & Kobayashi, K. (2007) Evaluation of variable numbers of tandem repeat as molecular epidemiological markers of *Mycobacterium tuberculosis* in Japan. *Journal of Medical Microbiology*, 56 (8): 1052-1057.

Wassink, G. J., Grogono-Thomas, R., Moore, L. J. & Green, L. E. (2003) Risk factors associated with the prevalence of footrot in sheep from 1999 to 2000. *Veterinary Record*, 152 (12): 351-358.

Wassink, G. J., Grogono-Thomas, R., Moore, L. J. & Green, L. E. (2004) Risk factors associated with the prevalence of interdigital dermatitis in sheep from 1999 to 2000. *Veterinary Record*, 154 (18): 551-555.

Wassink, G. J., King, E. M., Grogono-Thomas, R., Brown, J. C., Moore, L. J. & Green, L. E. (2010) A within farm clinical trial to compare two treatments (parenteral antibacterials and hoof trimming) for sheep lame with footrot. *Preventive Veterinary Medicine*, 96 (1-2): 93-103.

Whittington, R. J. (1995) Observations on the indirect transmission of virulent ovine footrot in sheep yards and its spread in sheep on unimproved pasture. *Australian Veterinary Journal*, 72 (4): 132-134.

Wickham, H. (2007) Reshaping data with the reshape package. *Journal of Statistical Software*, 21 (12): 1-20.

Wickham, H. (2009) *ggplot2: elegant graphics for data analysis*. New York: Springer-Verlag.

Winter, A. (2004a) Lameness in sheep 2. Treatment and control. *In Practice*, 26 (3): 130-139.

Winter, A. C. (2004b) Lameness in sheep 1. Diagnosis. *In Practice*, 26 (2): 58-63.

Winter, J. R., Kaler, J., Ferguson, E., KilBride, A. L. & Green, L. E. (2015) Changes in prevalence of, and risk factors for, lameness in random samples of English sheep flocks: 2004-2013. *Preventive Veterinary Medicine*, 122 (1-2): 121-128.

Witcomb, L. A. (2012) *The in situ analysis of the microbial community associated with footrot of sheep*. PhD thesis, University of Warwick, Coventry, UK.

Witcomb, L. A., Green, L. E., Calvo-Bado, L. A., Russell, C. L., Smith, E. M., Grogono-Thomas, R. & Wellington, E. M. H. (2015) First study of pathogen load and localisation of ovine footrot using fluorescence in situ hybridisation (FISH). *Veterinary Microbiology*, 176 (3-4): 321-327.

Witcomb, L. A., Green, L. E., Kaler, J., Ul-Hassan, A., Calvo-Bado, L. A., Medley, G. F., Grogono-Thomas, R. & Wellington, E. M. H. (2014) A longitudinal study of the role of *Dichelobacter nodosus* and *Fusobacterium necrophorum* load in initiation and severity of footrot in sheep. *Preventive Veterinary Medicine*, 115 (1-2): 48-55.

Yang, F., Zeng, X. W., Ning, K., Liu, K. L., Lo, C. C., Wang, W., Chen, J., Wang, D. M., Huang, R. R., Chang, X. Z., Chain, P. S., Xie, G., Ling, J. Q. & JianXu (2012) Saliva microbiomes distinguish caries-active from healthy human populations. *ISME Journal*, 6 (1): 1-10.

Yeruham, I. & Elad, D. (2004) Necrotizing stomatitis associated with *Fusobacterium necrophorum* in two goats. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health*, 51 (1): 46-47.

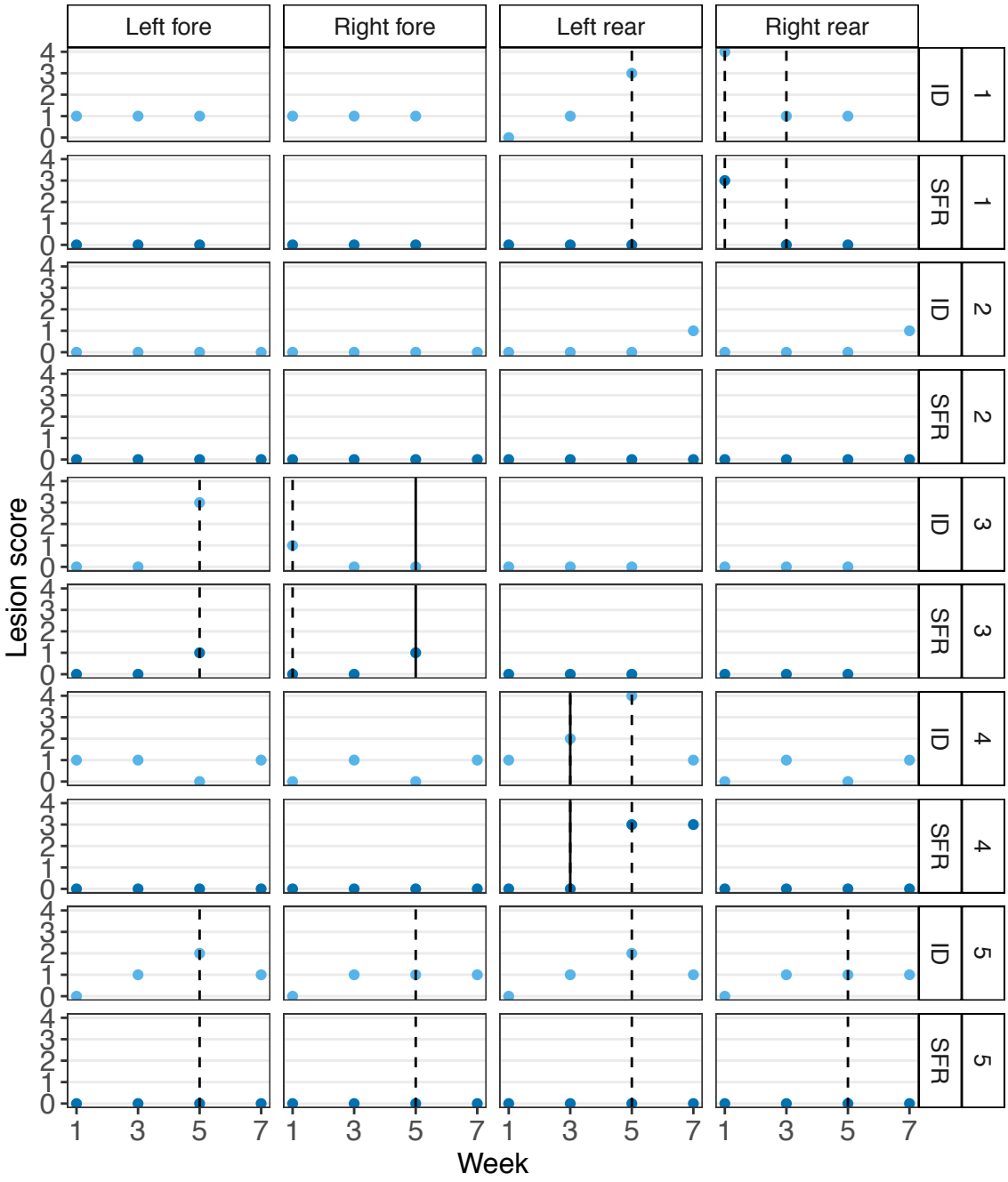
You, F. M., Huo, N. X., Gu, Y. Q., Luo, M. C., Ma, Y. Q., Hane, D., Lazo, G. R., Dvorak, J. & Anderson, O. D. (2008) BatchPrimer3: A high throughput web application for PCR and sequencing primer design. *Bmc Bioinformatics*, 9: 13.

Zadoks, R. N., Tikofsky, L. L. & Boor, K. J. (2005) Ribotyping of *Streptococcus uberis* from a dairy's environment, bovine feces and milk. *Veterinary Microbiology*, 109 (3-4): 257-265.

Zhou, H. T., Bennett, G. & Hickford, J. G. H. (2009) Variation in *Fusobacterium necrophorum* strains present on the hooves of footrot infected sheep, goats and cattle. *Veterinary Microbiology*, 135 (3-4): 363-367.

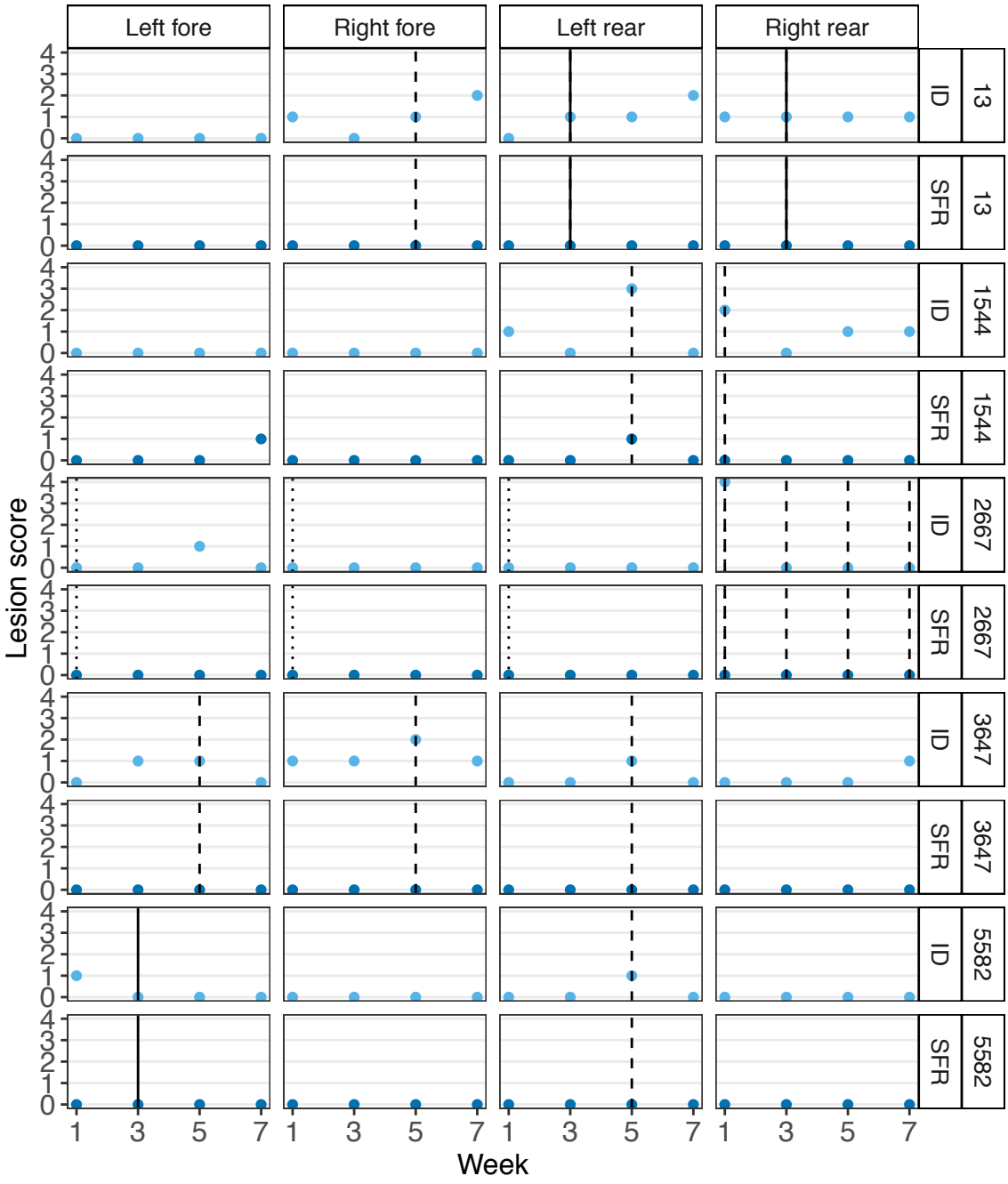
# Appendix 1: Individual lesion scores and treatments for sheep in Study A

**Lesion severity score and treatments over time for lambs in Study A**




Foot is indicated at the top of each column. Lesion severity axis is on the left. Lamb identification number (1-5) is shown on the right. Each animal has a panel for ID score and SFR score. Solid lines indicate foot trimming, and dashed lines indicate topical antibiotic spray.

**Lesion severity score and treatments over time for ewes in Study A**




Foot is indicated at the top of each column. Lesion severity axis is on the left. Ewe identification number is shown on the right. Each animal has a panel for ID score and SFR score. Solid lines indicate foot trimming, dashed lines indicate topical antibiotic spray and dotted lines indicate antibiotic injection.

# Appendix 2: Associations between variables tested in binomial mixed effects model of qPCR data from foot swabs in Study A

| | $Log_{10}$(Fn load + 1) | $Log_{10}$(Dn load + 1) | Time | Sheep positive on at least one foot | Lamb rather than ewe | Foot diseased | Antibiotic spray treatment | Trimming treatment | Mouth positive for Fn | Sheep diseased |
|---|---|---|---|---|---|---|---|---|---|---|
| $Log_{10}$(Fn load + 1) | | | | | | | | | | |
| $Log_{10}$(Dn load + 1) | | | | | | | | | | |
| Time | -0.56 | -0.27 | | | | | | | | |
| Sheep positive on at least one foot | + | + | - | | | | | | | |
| Lamb rather than ewe | + | - | | | | | | | | |
| Foot diseased | + | + | | | | | | | | |
| Antibiotic spray treatment | | + | + | a | | b | | | | |
| Trimming treatment | | | | | | | c | | | |
| Mouth positive for Fn | | | - | | d | | | | | |
| Sheep diseased | | | | | | e | f | | | |

**Continuous/ordinal associations**

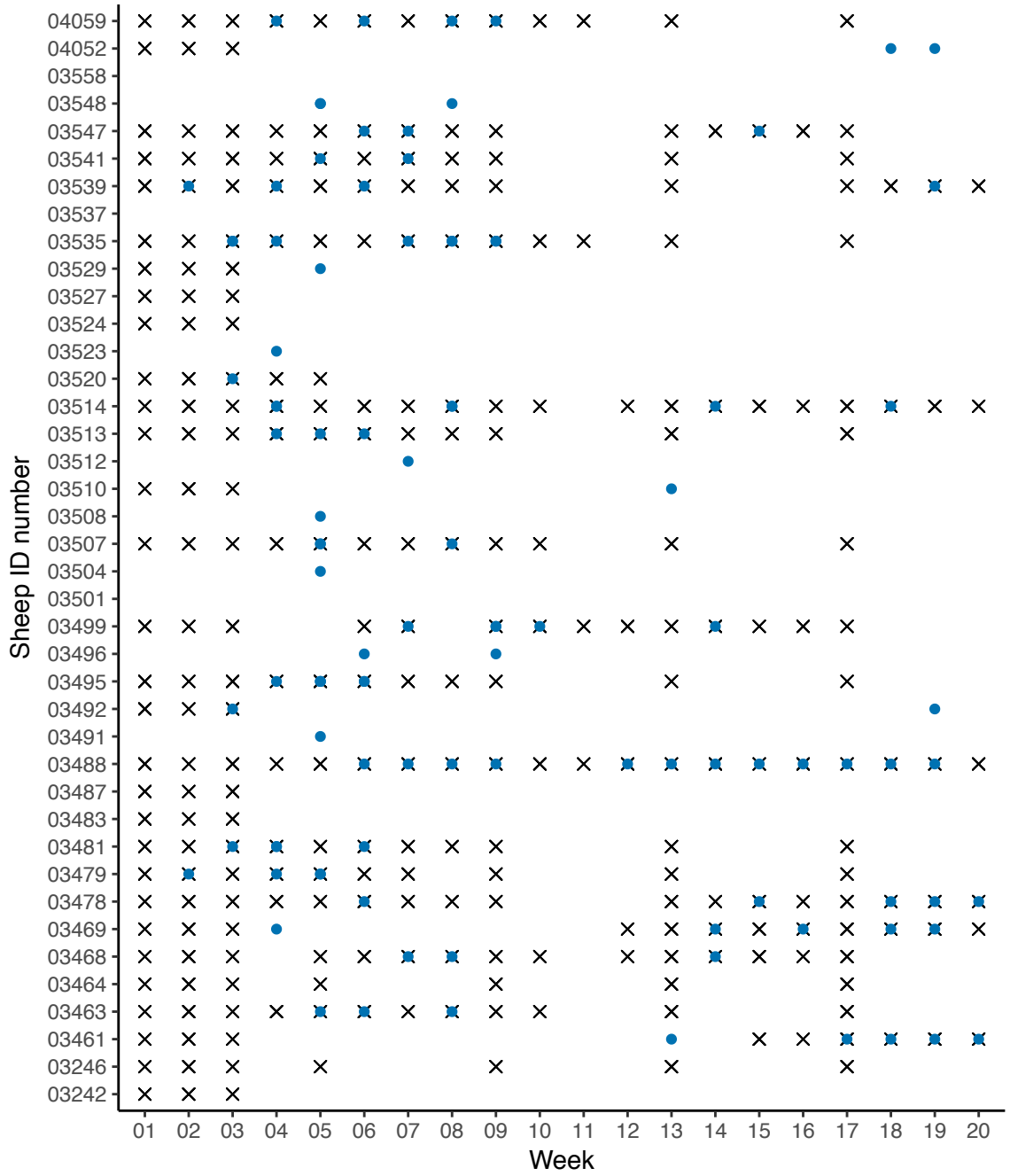
Spearman coefficients are given for significant correlations between two continuous variables. For correlations between a continuous/ordinal variable and a binary variable, + indicates that values for the continuous/ordinal variable are higher when the binary variable is true (= 1), - indicates that values for the continuous/ordinal variable are lower when the binary variable is true (= 1). Non-significant associations are not shown.

**Non-ordinal associations:**

**a**: treatment with antibiotic spray was associated with a sheep being negative for Fn on all four feet; **b**: treatment with antibiotic spray was associated with a foot being diseased; **c**: treatment with antibiotic spray was associated with trimming treatment; **d**: being a lamb was associated with having a mouth swab negative for Fn; **e**: a sheep being diseased was associated with a foot being diseased; **f**: a sheep being diseased was associated with antibiotic spray treatment.

# Appendix 3: Footrot occurrence by sheep, and sample selection, for Study B

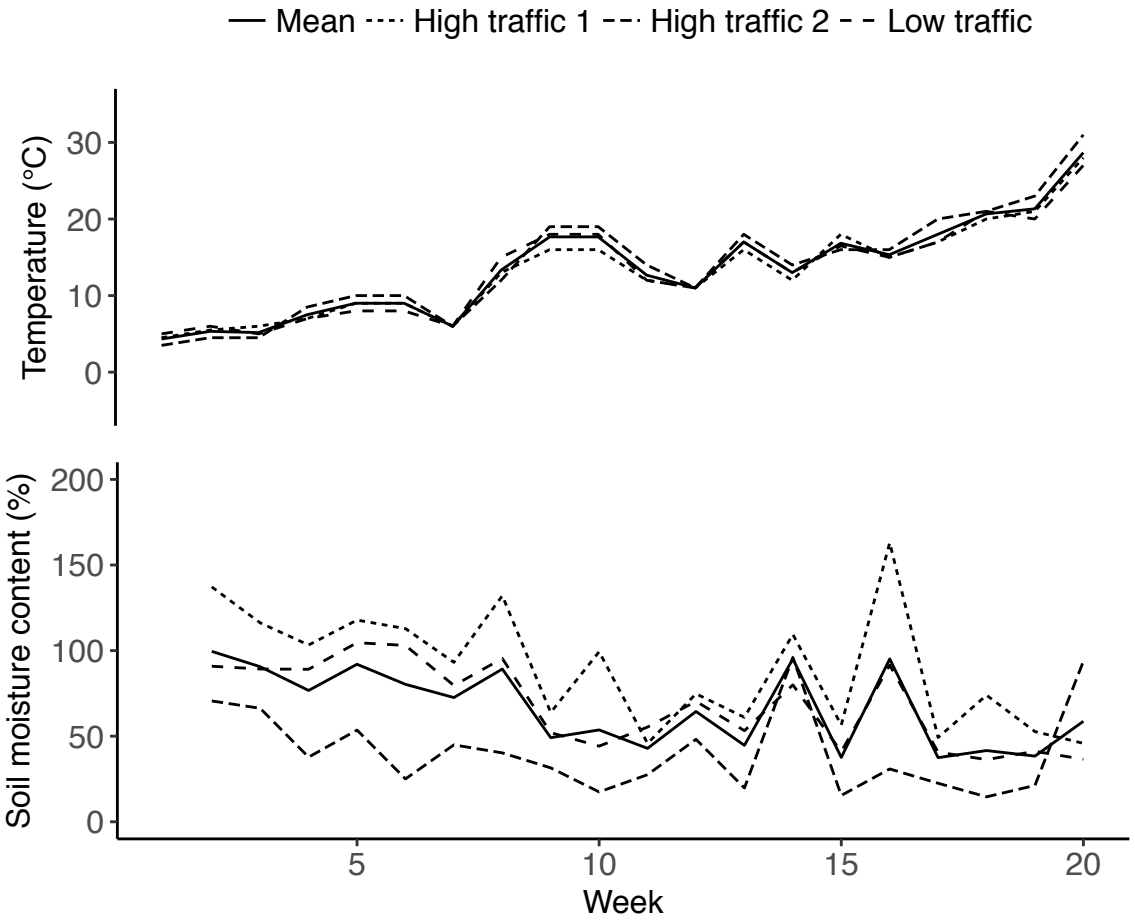
**Disease occurrence by individual sheep over time**




Blue circles indicate a sheep with at least one foot scoring ID > 1 and/or SFR > 0. Black crosses indicate sheep and weeks where samples were selected for analysis.

# Appendix 4: Soil moisture and soil temperature data for Study B

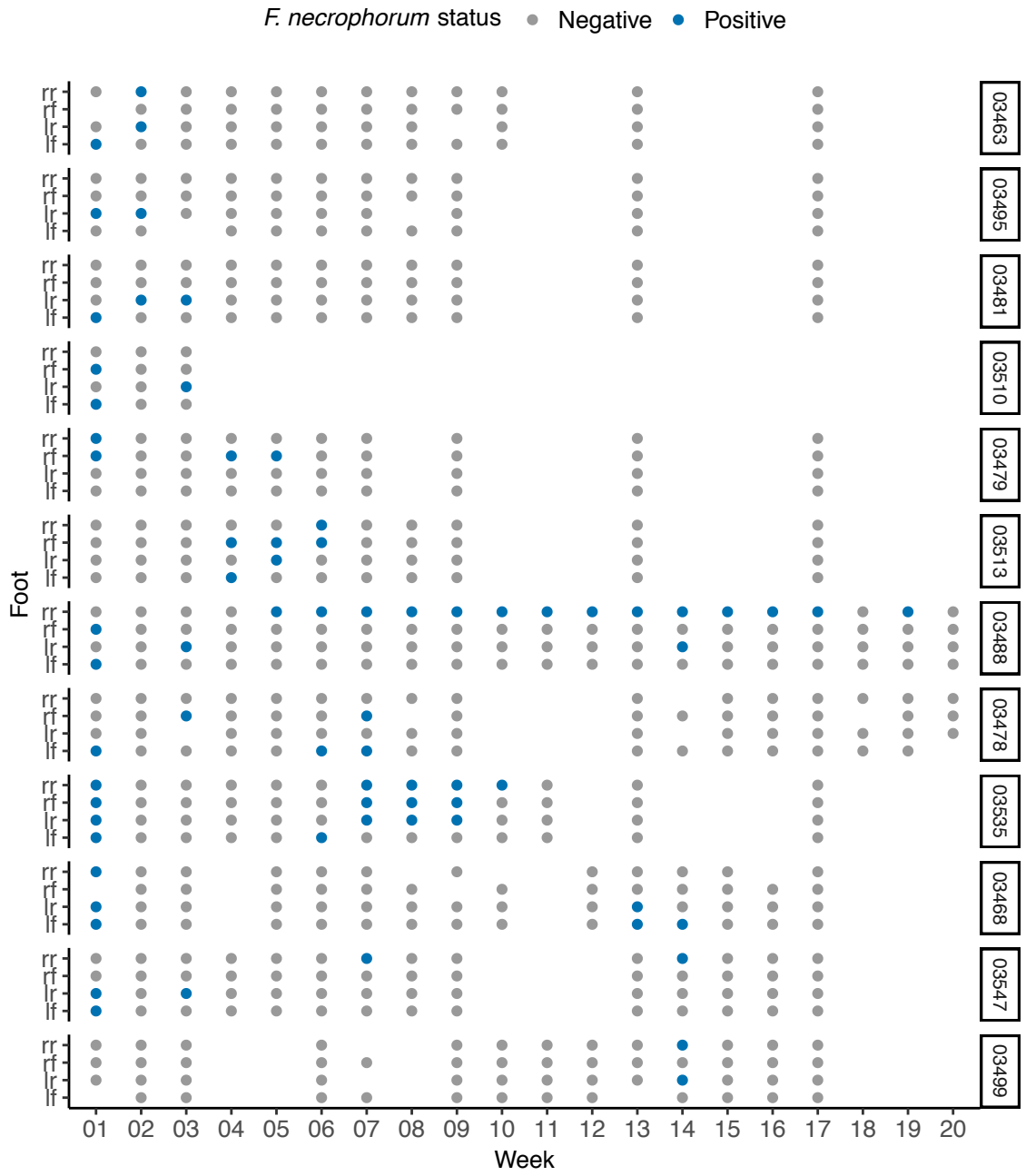
**Soil temperature and moisture data for Study B**




The weekly mean values (solid line) plus the values recorded for the 3 sampling locations are shown for soil temperature (upper panel) and soil moisture (lower panel).

# Appendix 5: Detection of *Fusobacterium necrophorum* on feet by sheep over time for Study B

**Detection of *F. necrophorum* by foot and sheep over time**




Only sheep with positive foot swabs for *F. necrophorum* after week 1 are shown. The numbers on the right of each panel are sheep ID number, and feet are shown on the *y* axis: lf = left fore, lr = left rear, rf = right fore, rr = right rear. Where results for only 2 or 3 feet are shown for a sheep on a particular week, other samples were omitted in the two batches of contaminated samples.

# Appendix 6: Associations between variables tested in binomial and linear mixed effects model of qPCR data from foot swabs in Study B

| | $Log_{10}$(Dn load + 1) | ID score | SFR score | Mean temp | Total rainfall | Soil moisture | Soil temp | Max temp | Min temp | % feet diseased | % feet Fn +ve | % mouths Fn +ve | % faeces Fn +ve | Foot diseased | Sheep diseased | Fn on ≥ 1 foot | Dn on ≥ 1 foot | Mouth Fn +ve | Faeces Fn +ve |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $Log_{10}$(Fn load+1) | 0.35 | | 0.24 | -0.11 | 0.12 | 0.11 | | | | -0.12 | 0.23 | 0.10 | | + | + | + | + | | |
| $Log_{10}$(Dn load+1) | | -0.07 | 0.14 | | 0.08 | | | | 0.08 | -0.08 | 0.10 | | -0.12 | + | | + | + | | - |
| ID score | | | 0.07 | | | | | | | 0.54 | | | 0.13 | + | + | | - | | |
| SFR score | | | | 0.15 | -0.13 | -0.15 | 0.17 | 0.15 | 0.09 | 0.17 | | | -0.10 | + | + | + | | | |
| Mean temp | | | | | | -0.69 | 0.9 | 0.95 | 0.81 | 0.27 | -0.34 | -0.53 | -0.62 | + | + | - | | - | - |
| Total rainfall | | | | | | 0.57 | -0.45 | | | -0.34 | 0.35 | 0.63 | 0.21 | - | - | + | + | + | |
| Soil moisture | | | | | | | -0.67 | -0.68 | | -0.31 | 0.22 | 0.42 | 0.42 | - | - | + | + | + | + |
| Soil temp | | | | | | | | 0.91 | 0.71 | 0.34 | -0.14 | -0.41 | -0.68 | + | + | - | | - | - |
| Max temp | | | | | | | | | 0.71 | 0.28 | -0.17 | -0.53 | -0.66 | + | + | - | | - | - |
| Min temp | | | | | | | | | | 0.08 | | -0.21 | -0.60 | + | + | | + | | - |
| % feet diseased | | | | | | | | | | | | 0.34 | 0.21 | + | + | - | - | | |
| % feet Fn +ve | | | | | | | | | | | | 0.61 | | | | + | | + | + |
| % mouths Fn +ve | | | | | | | | | | | | | 0.38 | | | + | | + | + |
| % faeces Fn +ve | | | | | | | | | | | | | | | - | - | - | + | + |
| Foot diseased | | | | | | | | | | | | | | | a | | | | |
| Sheep diseased | | | | | | | | | | | | | | | | b | | | |
| Fn on ≥ 1 foot | | | | | | | | | | | | | | | | | c | d | |
| Dn on ≥ 1 foot | | | | | | | | | | | | | | | | | | | e |
| Mouth Fn +ve | | | | | | | | | | | | | | | | | | | f |

**Continuous/ordinal associations**
Spearman coefficients are given for significant correlations between two continuous/ordinal variables. For correlations between a continuous/ordinal variable and a binary variable, + indicates that values for the continuous/ordinal variable are higher when the binary variable is true (= 1), - indicates that values for the continuous/ordinal variable are lower when the binary variable is true (= 1). Non-significant associations are not shown.

**Non-ordinal associations:**
**a**: a foot having footrot was associated with a sheep having footrot **b**: a sheep with footrot was more likely to have *F. necrophorum* on at least one foot; **c**: a sheep with *D. nodosus* on at least one foot was more likely to have *F. necrophorum* on at least one foot at the same time; **d**: a sheep with *F. necrophorum* on at least one foot was more likely to have a mouth swab positive for *F. necrophorum* the same week; **e**: a sheep with *D. nodosus* on at least one foot was less likely to have a faecal sample positive for *F. necrophorum* the same week; **f**: a sheep with a mouth swab positive for *F. necrophorum* was more likely to have a faecal sample positive for *F. necrophorum* the same week.

# Appendix 7: Tandem repeat regions identified in the *Fusobacterium necrophorum* genome and details of isolates used to test for polymorphism at these regions

**List of tandem repeat regions identified from *F. necrophorum* ATCC 51357 and results of selection criteria**

| Name | Sufficient flanking sequence for primer design | Flanking sequence in all 3 published Fn genomes | Positive on PCR for strain DSM 21784 | Polymorphism when tested with 8 Fn isolates |
|---|---|---|---|---|
| Fn1 | ✓ | ✓ | ✓ | |
| Fn2 | ✓ | ✓ | ✓ | |
| Fn3 | ✓ | ✓ | | |
| Fn4 | ✓ | | | |
| Fn5 | ✓ | | | |
| Fn6 | ✓ | | | |
| Fn7 | ✓ | | | |
| Fn8 | | | | |
| Fn9 | ✓ | | | |
| Fn10 | ✓ | | | |
| Fn11 | ✓ | | | |
| Fn12 | ✓ | | | |
| Fn13 | ✓ | ✓ | ✓ | ✓ |
| Fn14 | ✓ | ✓ | ✓ | |
| Fn15 | ✓ | ✓ | | |
| Fn16 | ✓ | ✓ | | |
| Fn17 | ✓ | ✓ | | |
| Fn18 | ✓ | | | |
| Fn19 | ✓ | | | |
| Fn20 | ✓ | | | |
| Fn21 | ✓ | | | |
| Fn22 | ✓ | ✓ | | |
| Fn23 | | | | |
| Fn24 | ✓ | | | |
| Fn25 | ✓ | ✓ | ✓ | |
| Fn26 | ✓ | ✓ | ✓ | |
| Fn27 | | | | |
| Fn28 | | | | |
| Fn29 | ✓ | | | |

| | | | | |
|---|---|---|---|---|
| Fn30 | ✓ | ✓ | ✓ | ✓* |
| Fn31 | | | | |
| Fn32 | | | | |
| Fn33 | ✓ | ✓ | ✓ | |
| Fn34 | ✓ | ✓ | | |
| Fn35 | ✓ | ✓ | ✓ | |
| Fn36 | ✓ | ✓ | ✓ | |
| Fn37 | ✓ | ✓ | | |
| Fn38 | ✓ | ✓ | ✓ | |
| Fn39 | ✓ | ✓ | ✓ | |
| Fn40 | ✓ | ✓ | ✓ | |
| Fn41 | ✓ | ✓ | ✓ | |
| Fn42 | ✓ | ✓ | ✓ | ✓ |
| Fn43 | ✓ | | | |
| Fn44 | ✓ | ✓ | ✓ | |
| Fn45 | ✓ | | | |
| Fn46 | ✓ | | | |
| Fn47 | ✓ | | | |
| Fn48 | ✓ | | | |
| Fn49 | ✓ | | | |
| Fn50 | ✓ | | | |
| Fn51 | ✓ | | | |
| Fn52 | ✓ | | | |
| Fn53 | ✓ | | | |
| Fn54 | | | | |
| Fn55 | ✓ | ✓ | ✓ | |
| Fn56 | ✓ | ✓ | ✓ | |
| Fn57 | ✓ | ✓ | | |
| Fn58 | ✓ | ✓ | ✓ | |
| Fn59 | ✓ | ✓ | | |
| Fn60 | ✓ | | | |
| Fn61 | ✓ | ✓ | ✓ | |
| Fn62 | ✓ | ✓ | ✓ | |
| Fn63 | ✓ | ✓ | ✓ | |
| Fn64 | ✓ | | | |
| Fn65 | ✓ | | | |
| Fn66 | ✓ | | | |
| Fn67 | ✓ | | | |
| Fn68 | ✓ | | | |
| Fn69 | ✓ | ✓ | ✓ | ✓ |
| Fn70 | ✓ | ✓ | 2 bands | |

| | | | | |
|---|---|---|---|---|
| Fn71 | ✓ | ✓ | ✓ | |
| Fn72 | | | | |
| Fn73 | | | | |
| **Total** | **64** | **34** | **24** | **3** |

* The primers for this locus demonstrated poor specificity when tested with non-target organisms and therefore this locus was excluded.

**Details of eight *F. necrophorum* isolates used to test for polymorphism at tandem repeat regions**

| Identification number | Site of origin | Country of origin |
|---|---|---|
| DSM21784 (Type strain) | Bovine hepatic abscess | Unknown |
| 02917023 | Sheep foot | France |
| 02919492 | | Spain |
| 02918013 | | Spain |
| 02917597 | | Spain |
| 02917818 | | Spain |
| 02917290 | | UK |
| 02920139 | | UK |

# Appendix 8: Details of strain types and community types identified for *Fusobacterium necrophorum*

**Twelve MLVA types identified among forty-six successfully typed isolates of *F. necrophorum***

| | Number of repeats | | | |
|---|---|---|---|---|
| MLVA type | Fn13* | Fn69* | Fn42 | Frequency |
| 1 | 2 | 2 | 5 | 10 |
| 2 | 2 | 4a | 3 | 1 |
| 3 | 2 | 3 | 5 | 9 |
| 4 | 1 | 3 | 6 | 12 |
| 5 | 1a | 2 | 2 | 5 |
| 6 | 2 | 4 | 5 | 2 |
| 7 | 2 | 3 | 6 | 2 |
| 8 | 2 | 4a | 5 | 1 |
| 9 | 2 | 2 | 6 | 1 |
| 10 | 1a | 4 | 4 | 1 |
| 11 | 1a | 3 | 6 | 1 |
| 12 | 1a | 4 | 6 | 1 |

*a=variant containing inserted DNA sequence within tandem repeat region

Six isolates were not successfully typed, either due to lack of amplification at one of the three loci (n=4), or due to the presence of two peaks on fragment analysis at one locus (n=2) suggesting the isolate may not have been axenic.

***F. necrophorum* community types from foot swabs (n=56), mouth swabs (n=32), and faeces (n=2) from sheep on 7 farms**

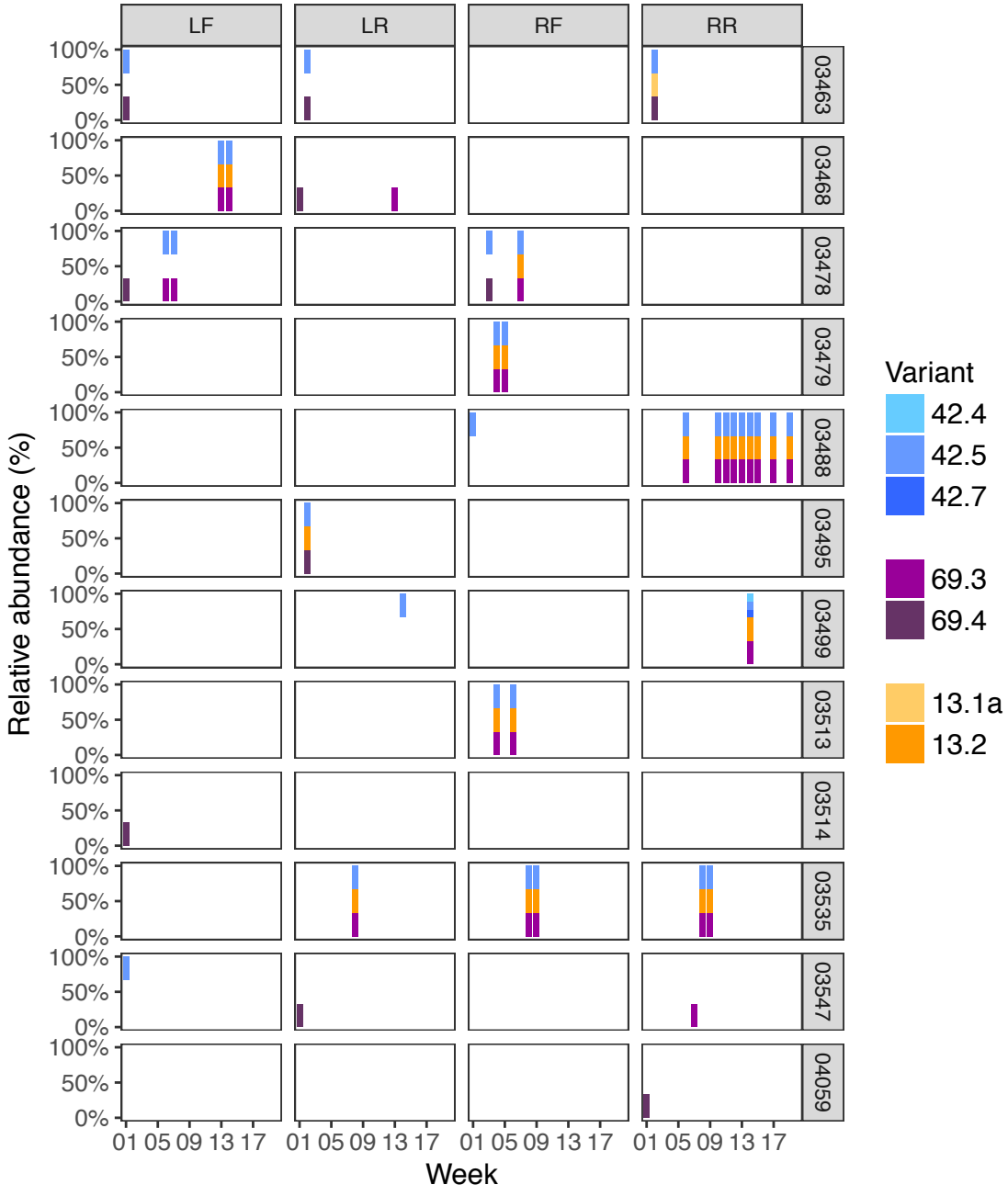
| | | | Variants present | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type | Site* | Frequency | Fn13.1a‡ | Fn13.2 | Fn69.2 | Fn69.3 | Fn69.4 | Fn42.1 | Fn42.2 | Fn42.3 | Fn42.4 | Fn42.5 | Fn42.6 | Fn42.7 | Fn42.8 |
| 1 | F,M&Fe | 34 | | 1 | 1 | | | | | | | 1 | | | |
| 2 | F&M | 3 | | 1 | 1 | | | | | 1 | | 1 | | | |
| 3 | M | 4 | 1 | 1 | 1 | | | | 1 | | | | | 1 | |
| 4 | M | 4 | | 1 | 1 | 1 | | | | | | 1 | 1 | | |
| 5 | M | 2 | 1 | | 1 | | | | | | | | 1 | | |
| 6 | M | 2 | 1 | | 1 | | | | | | | 1 | 1 | | |
| 7 | M | 1 | | 1 | 1 | | | | | 1 | | | | | |
| 8 | F,M&Fe | 27 | | 1 | | 1 | | | | | | 1 | | | |
| 9 | M | 2 | 1 | 1 | 1 | 1 | 1 | | | | | 1 | | | |
| 10 | M | 1 | | 1 | 1 | | | | 1 | | | | | | |
| 11 | M | 1 | 1 | 1 | 1 | 1 | | | | 1 | | | 1 | | |
| 12 | M | 1 | | 1 | 1 | 1 | | | | 1 | | | | | |
| 13 | M | 1 | 1 | | 1 | | | | | | | | 1 | 1 | |
| 14 | M | 1 | | 1 | 1 | 1 | | | | | | 1 | | | |
| 15 | M | 1 | 1 | | 1 | | | | | | | | | | 1 |
| 16 | M | 1 | | 1 | 1 | | | 1 | | | | 1 | | | |
| 17 | F | 2 | | 1 | | | 1 | | | | | 1 | | | |
| 18 | F | 1 | 1 | | | | 1 | | | | | 1 | | | |
| 19 | F | 1 | | 1 | | 1 | | | | | 1 | 1 | | 1 | |

* Site: F=foot, M=mouth, Fe=faeces

‡ a=variant containing inserted DNA sequence within tandem repeat region

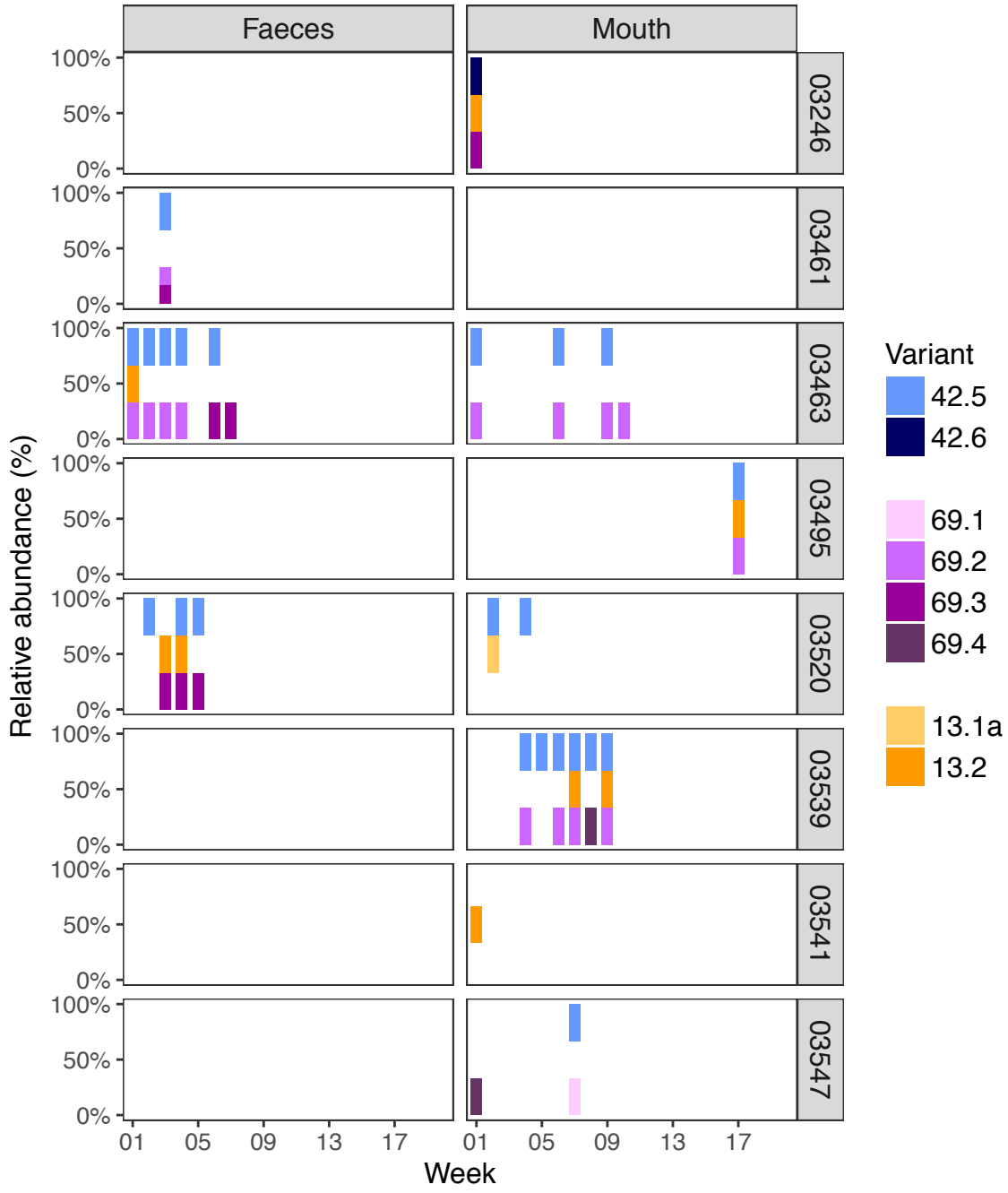
# Appendix 9: *Fusobacterium necrophorum* MLVA locus variants detected from foot swabs, mouth swabs and faecal samples from Study B

**Relative abundance of locus variants on foot swabs from Study B**




Foot is given at the top of each column (LF = left fore, LR = left rear, RF = right fore and RR = right rear). There is one panel for each sheep, and sheep ID number is given on the right of each panel.

**Relative abundance of locus variants in mouth and faecal samples from Study B**




Results from faecal samples are represented in the left-hand panels, and mouths in the right. There is one panel for each sheep, and sheep ID number is given on the right of each panel.

# Appendix 10: Additional publications

I published the following articles that do not contain material from this thesis during my PhD Studentship:

1. **Clifton R**., Green L. E., 2016. Pathogenesis of ovine footrot disease: a complex picture. *Veterinary Record*, 179, 225-227.
2. **Clifton R**., Green L. E., 2016. On the treatment, control and elimination of ovine footrot: a comparative review. *CAB Reviews,* 11 (053), 1-9.
3. **Clifton R**., Green L. E., 2017. Footrot in sheep: key messages from recent research. *Livestock,* 22 (3), 150-156.
4. **Clifton R**., Green L. E. (In Press). Managing footrot in sheep: an update. *In Practice.*